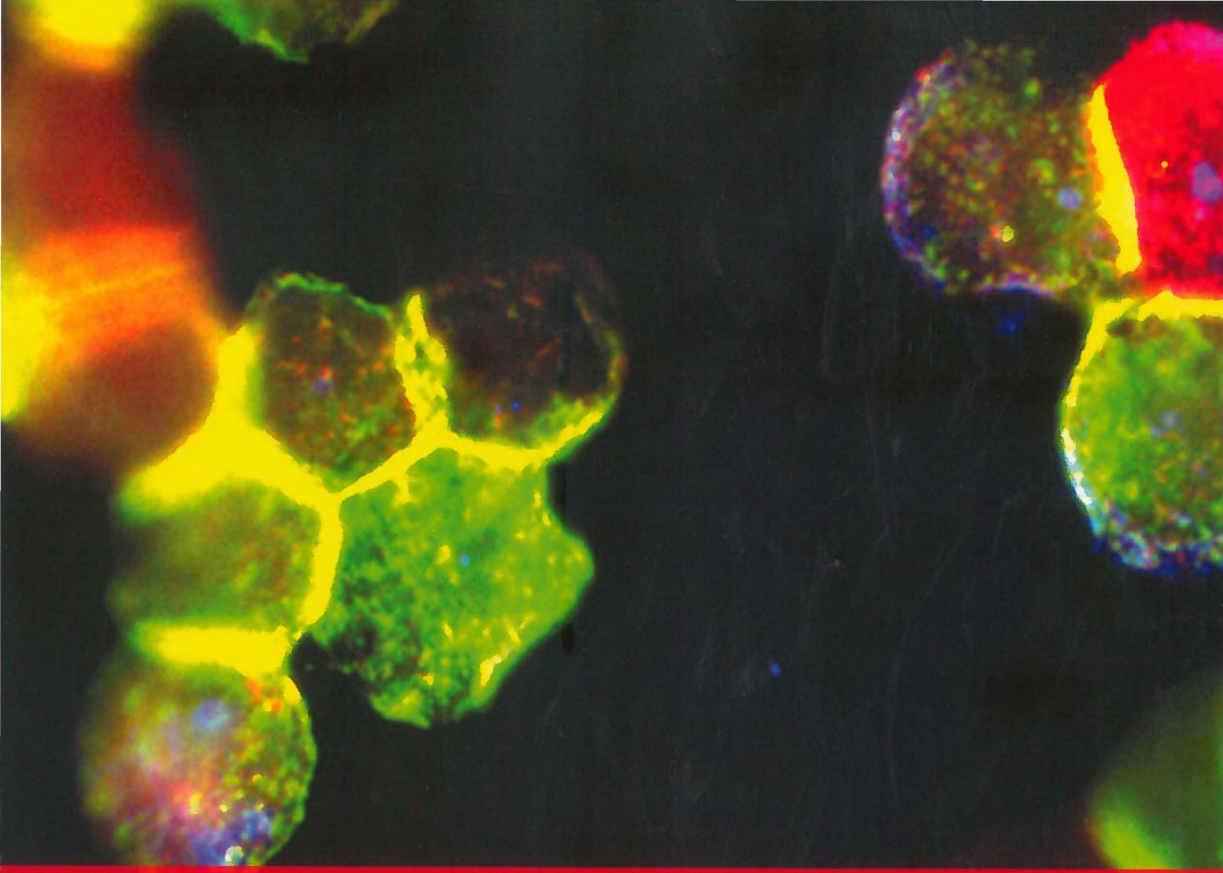
CD20 targeting and beyond

Stel, Aaltje Johanna



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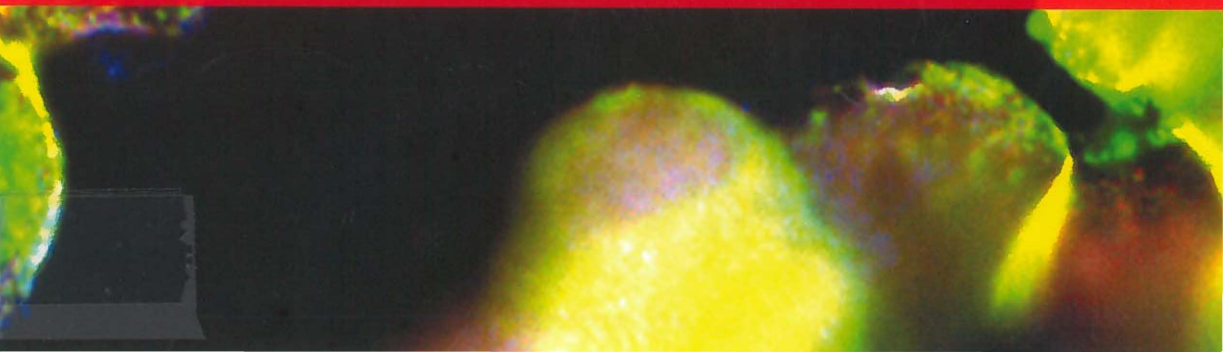
Alja Stel



CD20
Targeting and Beyond

A.J. Stel

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Stellingen behorende bij het proefschrift

CD20: targeting and beyond.

1. B-cellen zijn gunstige targets voor T-cel-gemedieerde therapie, via bijvoorbeeld BIS20x3, door de capaciteit van B-cellen om als antigeen presenterende cellen T-cel activatie te versterken via natuurlijke costimulatie (dit proefschrift).

2. BIS20x3 is als bispecifiek antilichaam tri-functioneel omdat het ten eerste T-cellen kan activeren en herrichten naar B-cellen, ten tweede B-cellen aanzet tot costimulatie van T-cellen en ten derde B-cellen gevoeliger maakt voor T-cel cytotoxiciteit en apoptose (dit proefschrift).

3. Translocatie van CD20 naar lipid rafts door binding van rituximab heeft als gevolg dat Fas spontaan kan clusteren waardoor de DISC gevormd wordt en caspases worden geactiveerd, uiteindelijk resulterend in death-receptor gemedieerde apoptose van de B-cel (dit proefschrift).

4. CD20 gebruikt niet alleen dezelfde signaleringsroute als HLA-DR maar kan ook een functionele interactie aangaan met HLA-DR, zodat een biologische functie van CD20 in de HLA-DR immuunsynaps waarschijnlijk wordt (dit proefschrift).

5. Gezien de colokalisatie van CD20 en de B-cel receptor, de functionele interactie tussen HLA-DR en CD20, en de spontane Fas clustering ten gevolge van CD20 activatie lijkt een biologische rol voor CD20 in de kiemcentra aannemelijk (J. of Immunology 2002;169:2886-2891 en dit proefschrift).

6. B-cel depletie middels rituximab in patiënten met een auto-immuunziekte als SLE of Reumatoïde Artritis heeft niet alleen een therapeutisch effect door afname van de productie van auto-antistoffen, maar lijkt in belangrijke mate effectief door het beïnvloeden van de immunologische interactie tussen B-cellen en T-cellen (J. of Immunology 2001;167:4710-4718 en Arthritis Rheum. 2005;52:501-513).

7. De toekomst van anti-CD20 therapie zal grotendeels bepaald worden door de ontwikkeling van volledig humane anti-CD20 antilichamen, aanpassingen in het Fc-domein van het antilichaam en het type anti-CD20 antilichaam (type I/II).

8. Het door de overheid voorgestelde snijden in de salarissen van specialisten in een maatschap maakt het werken in een academisch ziekenhuis financieel een stuk aantrekkelijker.

9. Het moeilijkste van het schrijven van een wetenschappelijk artikel is het schrappen van overbodige tekst.

10. Een proefschrift schrijven vereist hetzelfde als wat nodig is voor het winnen van een volleybalwedstrijd: het afmaken!

Alja Stel

Groningen, 27 okt 2010

CM6.

rijksuniversiteit groningen

CD20
Targeting and Beyond

PROEFSCHRIFT

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Voor mijn ouders en Marco

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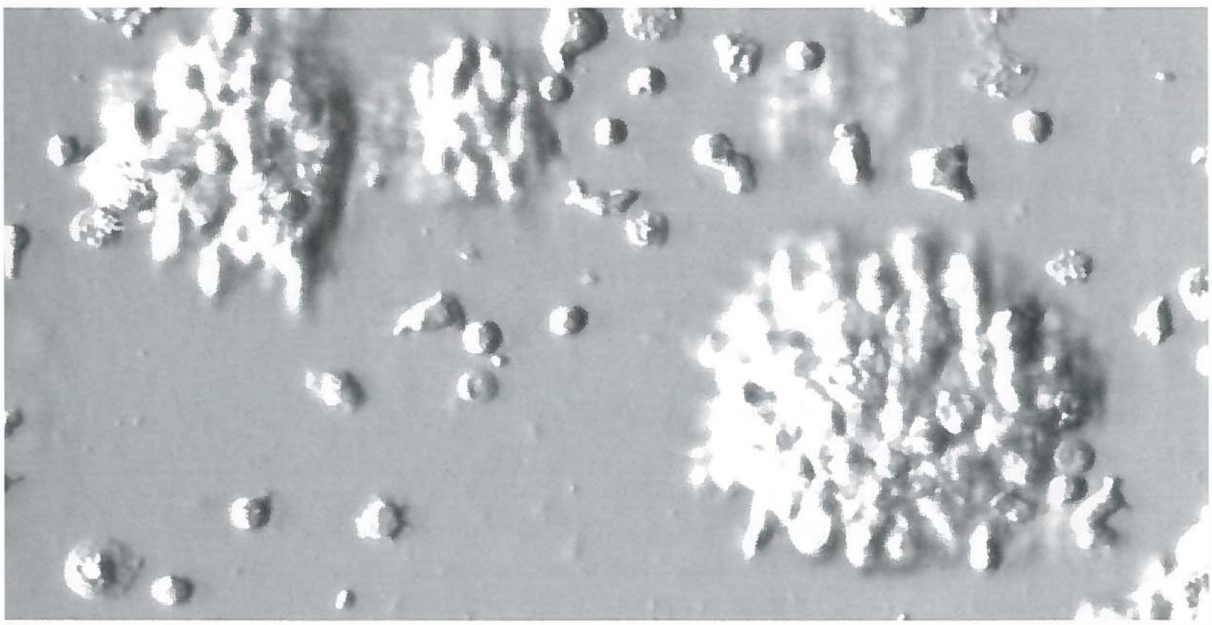

Introduction to the thesis.

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CD20: targeting and beyond.

Over the last 15 years, CD20 has proven to be an efficient target for antibody (Ab) mediated immunotherapy in treatment of Non-Hodgkin's lymphoma and various autoimmune diseases. Rituximab, a chimeric IgG1 mAb, was first approved by the FDA for use in relapsed low-grade or follicular B-cell lymphoma in 1997 (1). Treatment with rituximab induces depletion of B-cells by means of Ab dependent cellular cytotoxicity (ADCC), complement dependent lysis (CDC) and via direct apoptosis signaling (2). As a single agent, rituximab induced complete and partial responses up to 50-73% in patients with relapsed low-grade non Hodgkin's lymphoma and follicular B-cell lymphoma (3-5). However, rituximab is more often used in combination with chemotherapeutic regiments, of which CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone) is the most common used, resulting in 76-94% overall response rates (ORR) (6-11). Over the last few years, opportunities have increased for the clinical use of rituximab. Various B-cell mediated autoimmune disorders appeared to respond well to treatment with the CD20-targeting Ab. A rapid depletion of B-cells resulted in a promising clinical outcome in patients suffering from rheumatoid arthritis (RA), idiopathic thrombocytopenic purpura (ITP), ANCA-associated vasculitis, Sjögrens syndrome or systemic lupus nephritis (12-16).

Despite these clinical successes of rituximab and the experience obtained in the multiple trials performed until today, the question remains which mechanisms initiate and regulate the *in vivo* cell death effect of rituximab. Several groups described Fc-receptor positive cells and ADCC to be the most important effector mechanism in rituximab mediated cell death, whereas others reported the involvement of CDC to be the main effector mechanism in rituximab mediated cell death (17-20). The role of hypercrosslinking of CD20, the ensuing induction of CD20-mediated signaling cascades and the subsequent initiation of apoptosis is even less clear. Since the physiological role of CD20 on the membrane of B-cells of human origin is still far from elucidated, many research groups are working on revealing the CD20 structure, function, possible ligands, possible co-localizing complex molecules and various downstream signaling pathways.

This introduction, focuses on the current understanding of the CD20 molecule with regard to its structure, function, signal transduction pathways and associated proteins. Increasing our knowledge on molecular and functional aspects of CD20 could aid in the development of even more efficient CD20-based therapeutic modalities or synergistic combination therapy protocols for treatment of B-cell malignancies and autoimmune disorders.

CD20 and intracellular signaling

CD20 is a transmembrane phosphoprotein which, upon crosslinking into multimeric complexes, induced increased calcium conductance in B-cells and influences intracellular processes such as cell cycle progression and cell differentiation (21,22). The predicted amino acid sequence of CD20 revealed three major hydrophobic regions permitting the protein to pass through the cell membrane four times and locating both the amino- and carboxy-terminal domains within the cytoplasm (23,24) (see figure 1). CD20 has a molecular weight of 33, 34 or 35 kDa, depending on the phosphorylation status of the molecule at different sites. In resting B-cells CD20 appeared to be unphosphorylated, whereas proliferating B-cells, freshly isolated tumor cells or B-cell tumor cell lines expressed CD20 in the phosphorylated state (25). Its cytoplasmic domains are rich in serine and threonine residues, with multiple possible phosphorylation sites. However, CD20 has no intrinsic enzymatic activity. Instead, CD20-induced transmembrane signals appear to be regulated by protein kinases, e.g. protein kinase C PKC (23,25,26).

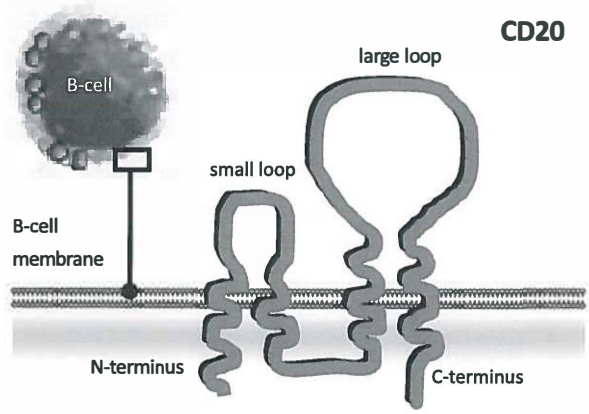

Figure 1. The CD20 molecule. CD20 is an integral membrane protein that crosses the cell membrane four times. It has two extracellular domains, one large loop and one smaller loop. Most antibodies targeting CD20 bind to the larger loop of the molecule.

Several cytokines are known to regulate kinase activity and may therefore influence CD20 phosphorylation, as described for LMW-BCGF (low molecular weight - B-cell growth factor) and INF-α in HCL (hairy cell leukaemia) cells (27). Additionally, HCL cells displayed high intracellular calcium levels concurrent with elevated phosphorylation of CD20, suggesting a link between the activation of calcium sensitive kinases and phosphorylation of CD20 (28). Transfection of CD20 cDNA into human T-cells or nonlymphoid cells induced an increased transmembrane calcium conductance in these cells and accelerated G_1 progression in a calcium entry dependent manner (21,22,29). The fact that specific immunoprecipitation of CD20 after crosslinking revealed homodimeric and homotetrameric clustering,

taken together with the described four-membrane spanning structure of CD20, led to believe that CD20 itself may form an calcium channel complex (21).
Alternatively, CD20 may be in close physical association with a calciumchannel-forming complex or calcium conductance is generated as a consequence of signal transduction via CD20, initiating a second messenger pathway regulating endogenous calcium channels (30). CD20 was also described to be involved in store-operated cation (SOC) entry of calcium and strontium, which is essential for regulating various cellular responses. This process was dependent on the association of CD20 with rafts for proper functioning of the channel and it was suggested that CD20 might be a functional component of such a SOC-entry pathway (30,31).

Deans *et al* demonstrated that CD20 ligation could also increase intracellular calcium independently of the opening of ion channels, via indirect activation of phospholipase C (32). The association of tyrosine and serine kinases with CD20 was followed by protein tyrosine kinase (PTK) activity and an increased mobilization of intracellular calcium. Specific association of the src-family tyrosine kinases Lyn, Fyn and Lck was found with CD20, which was independent of its cytoplasmic domains and appeared to be indirect, mediated by an undefined 75-80 kDa phosphoprotein (33,34). These results indicate an upstream signaling pathway initiated by CD20 crosslinking, involving the activation of src-kinases and calcium conductance. Src-protein kinases in turn can influence downstream B-cell signaling and development, for example via the regulation of NF-κB activity (35). CD20 stimulation by rituximab can inhibit constitutive NF-κB signaling in B-cell lymphoma cells, thereby controlling transcriptional activity within B-cells and sensitizing them to apoptosis (36,37). Other downstream signaling pathways, described to be under the influence of CD20 signaling, include the phosphoinositide-3-kinase (PI3K)/Akt pathway and the mitogen-activated protein kinase (MAPK) pathway. Akt is a serine/threonine protein kinase that mediates various signaling effects of PI3K. The activated PI3K-Akt pathway can provide survival signals to lymphoma cells by controlling a variety of mechanisms that inhibit apoptosis and prolong cell survival. Treatment of B-NHL cell lines with rituximab significantly inhibited the constitutively activated PI3K-Akt signaling pathway resulting in inhibition of both Bcl-xL activity and Bcl-xL expression (38). Both the PI3K inhibitor Ly294002 and the use of silencer (si)RNA for Akt inhibited this pathway and sensitized the cells to drug-induced apoptosis.

MAP kinases are a group of protein serine/threonine kinases that are activated by extracellular stimuli and transfer signals from the cell membrane towards the nucleus. The MAPK pathway can be divided into three major cascades, the ERK 1/2 (extracellular signal-related kinase) MAP kinase cascade, the JNK/SAPK (c-Jun

kinase/stress activated protein kinase) cascade and the p38MAPK cascade. NHL B-cell lines and freshly isolated tumor cells exhibit constitutively activated MAPK, NF-κB and Akt signaling pathways. Crosslinking of rituximab by anti-human IgG1 F(ab)2 fragments induced apoptosis in B-cell chronic lymphocytic leukemia (CLL) cells through a mechanism involving all three MAP kinases (39). Inhibition of p38MAPK activity completely blocked downstream signaling and reduced the degree of anti-CD20 induced apoptosis. Rituximab could inhibit constitutive p38MAPK or ERK 1/2 activity, decreasing transcriptional activity within various B-cell lines (40,41). Jazirehi *et al* generated rituximab-resistant clones of Ramos, Daudi and 2F7 NHL B-cell lines following extended culture of the cells in the presence of increasing concentrations of rituximab (42). These clones exhibited hyperactivation of the p38MAPK, NF-κB and ERK1/2 pathways.

Compared with the parental cells, the clones exhibited; 1.) significantly lower sensitivity to CDC-mediated killing; 2.) lower sensitivity to apoptosis induced by crosslinked rituximab and; 3.) higher levels of p-IκB-a, p-IKK, and p-ERK1/2 correlating with higher IKK and ERK1/2 kinase activities, leading to higher DNA-binding ability of AP-1 and NF-κB, eventually culminating in increased Bcl-2, Mcl-1, and Bcl-xL expression. The downregulation of the anti-apoptotic gene products and inhibition of survival pathways by rituximab were responsible for chemosensitization of drug resistant B-NHL cell lines (43). It appears from all these data that CD20 can influence B-cell signaling and gene transcription via various mechanisms and cascades, including calcium mobilization, activation of src-kinases and regulation of MAPK, NF-κB and Akt survival pathways, all together orchestrating the cells destiny.

CD20-mediated apoptosis

Early reports on engagement of CD20 by monoclonal Abs demonstrated rescue from anti-BCR-induced programmed cell death or suppression of spontaneous apoptosis which normally occurs in human germinal center (GC) B cells (44,45). However, Shan *et al* described in 1998 that hypercrosslinking of CD20 via either secondary goat-anti-mouse Abs or FcR-bearing cells, could induce apoptosis in B-cell lines and normal tonsil B-cells (46). Apoptosis can be measured by many different essays. Most of them are based on specific apoptosis features, such as blebbing, loss of membrane integrity, cell shrinkage, DNA fragmentation, loss of mitochondrial potential or activation of specific apoptosis-dependent proteins like caspases (47). Shan *et al* presented DNA fragmentation by propidium iodide (PI)

staining, DNA-laddering, and typical apoptotic scattering upon B-cell treatment with CD20 Abs B1 and 1F5 when crosslinked using secondary crosslinking Abs. For an overview of anti-CD20 Abs used to investigate CD20 signaling, see table I. In 2000, they showed that calcium fluxes are involved in efficient induction of cell death via rituximab, B1 and 1F5 (48). The classic substrate for caspases, PARP (poly ADP-ribose polymerase) was cleaved and addition of the caspase inhibitor zVAD decreased both PARP cleavage and DNA-fragmentation implying that apoptosis was induced in a caspase dependent manner.

Similarly to anti-BcR-induced apoptosis, CD20 clustering was thought to induce the classical intrinsic/mitochondrial pathway, via cytochrome-c release and caspase-9 activation (57). *In vivo* data from patients with CLL, receiving rituximab treatment indeed showed activation of caspase-9, caspase-3 and PARP cleavage in blood leukaemia cells (58). Next to the mitochondrial apoptosis pathway, other routes were also proposed to be involved in CD20 mediated apoptosis. CD20 engagement induced the activation of caspase-8, the executioner caspase of the death receptor apoptosis pathway, in several B-cell lines and in patient material (59,60).

Table I: overview of anti-CD20 Abs used to investigate CD20 signaling

CD20 Ab	isotype	apoptosis		raft translocation	type I/II	ref
		- CL	+ CL	- / +		
B1	m-IgG2a	+	+	-	II	Stashenko [49]
rituximab	hu-chim-IgG1	-	+	+	I	Reff [50]
1F5	m-IgG2a	-	+	+	I	Clark [51]
2H7	m-IgG2b	-	+	+	I	Clark [51]
B-ly1	m-IgG1	-	+	+	I	Poppema [52]
7D2	m-IgG1	-	+	+	I	Teeling [53,54]
2F2	hu-IgG1	-	+	+	I	Teeling [53,54]

m = murine, hu = human, chim = chimeric, CL = crosslinking

Other CD20 Abs, like 2H7, induced caspase activation as well (55). These results suggest a signaling pathway emanating from CD20 hypercrosslinking which results in cleavage of caspases and the induction of apoptosis. This is similar to the signaling cascade induced by B-cell receptor (BcR or IgM) engagement, involving cleavage of caspase-3, upregulation of the pro-apoptotic protein Bax, changes in mRNA levels of c-myc and Berg36, activation of MAPKs p44 and p42 (extracellular

signal-regulated kinase-1 and -2) and activation of the DNA-binding capacity of activator protein 1 (AP-1) (56).

In this thesis we demonstrate that the death receptor pathway also plays a significant initiating role in CD20-mediated apoptosis (61). Apart from the original, mitochondrial pathway and the newly identified involvement of the death receptor pathway, other reports showed that CD20-mediated apoptosis could also occur independent of both the loss of mitochondrial membrane potential and the activation of caspases (62). In Ramos cells displaying relative resistance to either Fas or BcR mediated apoptosis, CD20 crosslinking still induced cell death, as measured by Annexin-V binding and PI uptake, even though mitochondrial membrane potential loss and cytochrome-c release were prevented. The pan-caspase inhibitor Z-VAD inhibited, in these experiments, caspase-3 cleavage (partially), caspase-9 cleavage and processing of PARP, suggesting an alternative pathway for the induction of apoptosis via CD20 hypercrosslinking. One possibility might involve the generation of reactive oxygen species. Bellosillo *et al* described a caspase-independent mechanism of rituximab mediated cytotoxicity, where rituximab in the presence of human serum induced a rapid production of reactive oxygen species, accompanied by PI uptake and loss of mitochondrial membrane potential, but not inhibited by caspase inhibitors (63). Recently, another mechanism for CD20 Ab-mediated cell death was proposed, acting via an autophagic pathway of cell destruction. This pathway promotes a cytoplasmic form of cell death, involving lysosomes, which swell and then disperse their contents, including cathepsin B, into the cytoplasm and surrounding environment. The resulting loss of plasma membrane integrity appeared to occur independently of caspases and was not controlled by Bcl-2 (64). The above described data suggest various mechanisms upon which CD20 crosslinking can lead to apoptosis, and probably even more routes are involved. Calcium fluxes could for example lead to damage to the endoplasmic reticulum (ER) membrane and activation of ER linked caspases, as reviewed by Rao at al (65). No evidence for CD20 involvement in ER membrane remodelling has been published so far, but it is clear that CD20 triggering and crosslinking can induce cell death via various different apoptosis signaling pathways.

Ever since apoptosis was reported upon triggering of the CD20 molecule, discussion has been going on if hypercrosslinking via secondary Abs or effector cells is imperative for the execution of cell death. In Daudi cells, rituximab alone was sufficient to induce early apoptosis as demonstrated by phosphatidyl serine expression on the outside leaflet of the membrane. After 24 hours of culture late (complete) apoptosis was shown by uptake of PI in 75% of the cells (66). In Ramos

cells, apoptosis could be induced using rituximab, B1 and 1F5, but was significantly increased by adding goat-anti-human or goat-anti-mouse Abs (48). Cardarelli *et al* showed that in BALL-1 cells, B1 could induce apoptosis without crosslinking, whereas rituximab in the same concentration did not (67). Crosslinking of rituximab with a secondary goat-anti-human Ab increased levels of apoptosis. These findings were confirmed by Cragg and Glennie in EHRB-Ramos, Daudi and Raji cells, except that in their experimental setting non-crosslinked rituximab could also induce minor levels of apoptosis in Daudi cells (68). Additionally, homodimers of rituximab, but not monomers, induced apoptosis in various cell lines, among which Ramos, Daudi, Raji, SU-DHL-4 and Namalwa, an effect that could be inhibited by caspase inhibitors (69). An extensive overview concerning these conflicting findings was published in 2002 by Julie Deans *et al* (70). In table II on overview is presented of all cell lines mentioned, described or used in this thesis.

Differences between results in different laboratories and in different cell lines might be caused by variation in cell culture conditions and cell cycle stage. However, recent reports show that it is likely that an important part of the described variation might be related to the selection of the type of anti-CD20 Ab. Most groups have studied the so called type-I or rituximab-like anti-CD20 Abs, which can activate complement, but are relatively poor in inducing apoptosis unless hypercrosslinked by secondary Ab. The rarer type II, or tositumomab-like anti-CD20 Abs appear less efficient in complement activation, but tend to promote apoptosis as IgG or F(ab')2 molecules (68,88,89).

Table II: overview of cell lines described / used in this thesis

Cell line	species	morphology	origin	commercial source	ref
Ramos	human	B-lymphoblast	Burkitt'slymphoma	ATCC: CRL-1596	Klein [71]
Daudi	human	B-lymphoblast	Burkitt's lymphoma	ATCC: CCL-213	Klein [72]
BALL-1	human	B-lymphoblast	acute lymphoblastoid leukemia	JCRB: 0071	Miyoshi [73]
Raji	human	B-lymphoblast	Burkitt's lymphoma	ATCC: CLL-86	Pulvertaft [74]
JY	human	B-lymphoblast	Epstein Barr virus-transformed lymphoblast cells	ATCC: 77441	Reiss [75]
BJAB	human	B-lymphoblast	Epstein Barr virus-transformed Buritt's lymphoma	ATCC: HB 136	Menezes [76]
SU-DHL-4	human	B-lymphoblast	diffuse histiocytic lymphoma	DSMZ: ACC 495	Epstein [77]
2F7	human	B-lymphoblast	AIDS associated Burkitt's lymphoma	ATCC: CRL-10237	Ng [78]
Namalwa	human	B-lymphoblast	Burkitt's lymphoma	ATCC: CRL-1432	Klein [79]
DoHH-2	human	B-lymphoblast	immunoblastic B cell lymphoma with the 14;18 chromosomal translocation	DSMZ: ACC 47	Kluin-Nelemans [80]
ALL-CM	human	B-lymphoblast	acute lymphoblastoid leukemia	not commercially available	Nijmeijer [81]
BLS-2	human	B-lymphoblast	Epstein Barr virus-transformed bare lymphocyte syndrome	not commercially available	Hume [82]
AML-193	human	monocyte	acute myeloid leukemia	DSMZ: ACC 549	Lange [83]
U-937	human	monocyte	diffuse histiocytic lymphoma	ATCC: CRL-1593.2	Sundström [84]
Jurkat	human	T-lymphoblast	acute lymphoblastic T-cell leukemia	DSMZ: ACC 282	Schneider [85]
CTLL-2	mouse	T-lymphoblast	lymphocyte cytotoxic T lymphocyte	ATCC: TIB-214	Gillis [86]
A20	mouse	B-lymphoblast	reticulum cell sarcoma	ATCC: TIB-208	Kim [87]

ATTC = American Type Culture Collection , JCRB = Japanese Collection of Research Bioresources, DSMZ = German Collection of Microorganisms and Cell Cultures

Type I Abs are demonstrated to transmit their apoptotic signal dependent on CD20 translocation into lipid raft microdomains, where CD20 becomes associated with the BCR (90,91). Walshe *et al* demonstrated recently that type I Abs invoke increases in cytosolic calcium levels, resulting from the direct association between CD20 and the BcR, as measured by fluorescence resonance energy transfer (FRET), resulting in the phosphorylation of BcR-specific adaptor proteins such as B-cell linker (BLNK) and SLP-76 (92). No such association with the BcR is produced by type II Abs, which is in line with their inability to evoke calcium influx and CD20 translocation into lipid rafts.

Thus, it appears that CD20-mediated apoptosis can involve several pathways, either caspase dependent or independent and requires hypercrosslinking depending on the used cell line and type of Ab (type I or II). Other CD20 targeting associated characteristics, like complement activation, calcium influx, BcR association and raft translocation also appear to depend on the Ab used, making it very interesting to study type I and II CD20 Abs *in vitro* and *in vivo* for their clinical relevance.

CD20 association with lipid rafts

In immune cell signaling, lipid rafts are essential platforms for receptor signaling. Lipid rafts are microdomains in the plasma membrane that contain high concentrations of cholesterol and glycosphingolipids. They compartmentalize cellular processes by serving as organizing centers for the assembly of signaling molecules, influencing membrane fluidity and membrane protein and receptor trafficking. Not all rafts are identical in terms of the proteins or the lipids that they contain. A variety of proteins, especially those involved in cell signaling, have been shown to partition into lipid rafts. For example, calcium channels are associated with these microdomains (93). In 1998, Deans *et al* showed for the first time that upon binding of CD20 Abs, CD20 was redistributed to lipid rafts, as determined by Triton-insoluble, low density membrane compartments (94). Within 15 seconds of Ab incubation, up to 95% of all CD20 molecules were translocated into lipid domains.

A membrane-proximal sequence in the carboxyl terminus of the CD20 molecule was identified to be essential for redistribution of CD20 to rafts. Deletion of the residues 219-225 induced a 75% decrease of translocation of CD20 into lipid rafts (95). It was suggested that phosphorylation of CD20 at these residues might be

required for raft translocation and/or that these specific sequences in the cytoplasmic carboxyl terminus of CD20 may be involved in promoting oligomerization of CD20, thereby facilitating its association with rafts. Additional crosslinking of CD20 Abs was not necessary for the redistribution into rafts (96). The capacity of anti-CD20 Abs to redistribute CD20 into rafts appears important for efficient tumour cell depletion, both on the level of intracellular signaling, and in the capability to activate lytic complement. It was shown that the majority of CD20 Abs (among which; rituximab, B1, B-ly1 and 2H7) all recognize the same extracellular epitope containing the alanine residue at position 170 and the proline residue at position 172 being the critical determinants (97). Abs for the 2H7 epitope needed additional residues on positions 162-166 and further depended on the presence of CD20 in an oligomeric complex. Different from the other Abs, the Ab 1F5 is described to recognize the second extracellular epitope of CD20 and is therefore considered unique among CD20 Abs. The characteristics of rituximab, B1, 1F5 and 2H7 were compared and reviewed by Deans *et al*, describing rituximab and 2H7 to be more raft-associated as compared to 1F5 or B1. This correlated with src-family tyrosine kinase activity and Deans *et al* suggested that the apoptotic response to CD20 Abs results from transactivation of the src-family of tyrosine kinases upon clustering of lipid rafts (70).

Rituximab treatment of B-cell lines was also responsible for a rapid and transient increase in cellular ceramide concentration and co-localization of ceramide with CD20 in rafts (98). Depletion of cholesterol resulted in total inhibition of ceramide generation and CD20 raft translocation (98,99), implying that cholesterol-dependent integrity of lipid rafts is necessary for efficient CD20 redistribution, stimulation of ceramide and probably for other CD20-triggered downstream effects as well. Finally, also complement-mediated lysis has been described to be correlated with translocation of CD20 into lipid rafts. CD20 Abs that were unable to segregate CD20 into rafts (type II Abs) failed to lyse cells in CDC assays (89). The CDC-inducing activity of type I raft-inducing CD20 Abs as rituximab and 1F5 was mediated by efficient C1q binding and the capacity to deposit C3b onto target cells, a process that failed when the type II non-raft inducing Ab B1 was used (89). The translocation of CD20 into lipid rafts appeared essential for the type I Ab to bind C1q and activate complement. This is probably because the process concentrates the Ab Fc-regions in such a way that they very efficiently engage multiple globular heads of the C1q molecules, thereby initiating the classical complement pathway (53,54). In addition, at saturation, the number of type I Abs bound to each B-cell was twice that observed for type II Abs (53). A possible explanation for these differences in binding stoichiometry may be that type II mAbs engage two adjacent extracellular loops within a complex whereas the type I mAbs bind only one.

However, while this explanation might satisfy the observed 2:1 binding-ratio, it does not explain differences in the induction of CD20 translocation into lipid rafts between type I and II Abs, nor does it explain why only type I Abs associate with the BcR in rafts. Co-localization of the BcR and CD20 after receptor ligation was rapidly followed by activation-dependent dissociation into distinct lipid rafts (90). It was suggested that by means of this process, selective internalization of the BcR after stimulation was distinguished from other raft-associated proteins, like CD20, which remain on the surface. It was proposed that CD20 may use the BcR signaling pathway to hijack downstream signals triggered by the BcR (92,100).

CD20 and sensitization

Rituximab treatment has proven to be very successful as a single agent therapy, but over the last few years it is also frequently used in combination with various types of chemotherapy. As described above, rituximab is most often combined with CHOP, resulting in high ORRs (6,7,101). In a nine years follow-up study performed by Czuczman *et al*, patients with follicular lymphoma displayed a 100% ORR with a median time to progression of 82.3 months (102). Numerous other regimens are currently under research for combined treatment with rituximab (103-108). It is suggested that combining rituximab with chemotherapeutic agents not only results in the additive effect of both treatment modalities, but rituximab can also sensitize lymphoma cells to cytotoxic drugs, which is especially beneficial in chemoresistant or refractory malignancies. For example, R-CHOP was associated with a significantly better survival in Bcl-2 positive diffuse large B-cell lymphoma patients as compared to CHOP alone, an effect not seen in Bcl-2 negative patients (109). This suggested rituximab to inhibit the tumor-protecting effect of Bcl-2, thereby preventing treatment failure. Downmodulation of other anti-apoptotic proteins like XIAP or Mcl-1 by rituximab was also observed, making lymphoma cells more prone to chemotoxic effects (58).

Sensitization by rituximab was described not to be dependent on complement, since application of complement in addition to rituximab did not further decrease the IC_{30} or IC_{50} (inhibitory concentration; dosage of drug necessary to induce 30% or 50 % apoptosis) of any of the tested chemotherapeutic agents (60). Chemosensitization was also independent of Fc-function, since rituximab (Fab')2 fragments and rituximab Abs devoid of the CH2 Fc-binding domain both sensitized resistant B-NHL cells to Cisplatin and Fas ligand-induced apoptosis, identical to the parental rituximab Ab (110). In the same study, treatment of nude mice bearing

Raji tumourcell xenografts with the combination of rituximab (Fab')2 or rituximab and Cisplatin resulted in similar and significant inhibition of tumor growth.

Bonavida *et al* extensively studied the mechanisms by which rituximab-mediated chemosensitization takes place at a cellular level, as reviewed in 2007 (43). They discovered that activation of CD20 via rituximab influences several signaling cascades within B-cells. Tumor B-cells synthesized and secreted anti-apoptotic cytokines like IL-10, working in an autocrine/paracrine loop that could be downregulated by rituximab treatment in several B-cell lines (111). The mechanism by which rituximab regulated IL-10 expression was found to involve inhibition of the phosphorylation state of the src-family tyrosine-kinase Lyn, leading to inhibition of the p38MAPK signaling pathway and decreased transcriptional activity of Sp1, a transcription factor responsible for IL-10 transcription and secretion (40). Downregulation of IL-10 secretion influenced the JAK/STAT (Janus Kinase/Signal Transducer and Activator of Transcription) pathway by inhibition of STAT3 activity, resulting in subsequent downregulation of Bcl-2 and sensitization to chemotherapeutic drugs (112). This selective dowregulation of Bcl-2 was also involved in the sensitization of the lymphoma cell line 2F7 to Cisplatin (113). In this study, rituximab could modify the cisplatin-mitochondrial signaling pathway in such a way that mitochondrial-mediated apoptotic events like membrane potential loss, cytochrome c release and caspase activation were induced in a syngergistic manner upon combinational treatment. It appeared that the down-regulation of Bcl-2 was an early event in rituximab treatment and was followed by decreases in additional anti-apoptotic proteins like cIAP-1, cIAP-2 and XIAP, enhancing the chemosensitizing effect of rituximab.

Another mechanism of sensitization was described by Jazirehi *et al*, reporting that rituximab could selectively decrease Bcl-xL expression and sensitized B-cell lines to paclitaxel-induced apoptosis via regulation of the ERK 1/2 pathway (41,114). Specifically, rituximab appeared to reduce the phosphorylation state of components of the ERK 1/2 signaling pathway through upregulation of RKIP (Raf-1 kinase inhibitor) expression. In accordance with its name, RKIP blocked phosphorylation and activation of Raf-1, one of the central kinases in the ERK 1/2 pathway. Upregulation of RKIP by rituximab resulted in inhibition of the constitutively active ERK 1/2 pathway, affecting activator protein-1 (AP-1) DNA binding activity and reducing Bcl-xL expression, eventually leading to chemosensitization of NHL B-cells (41). Similar to the ERK 1/2 pathway, the NF-κB pathway is also constitutively activated in mature B-cells and B-cell lymphomas (115,116). Rituximab-mediated upregulation of RKIP appeared not only to block Raf-1 phosphorylation, but also inhibited members of the NF-κB pathway, like NIK

(NF-κB-Inducing-Kinase), TAK1 (TGF-β Activating Kinase 1) and (IκB Kinase) IKK (37,117). This led to an inhibition of NF-κB activity in these cells, resulting in decreased transcription of genes that are under the control of NF-κB, like Bcl-2 or Bcl-xL. For an overview of pathways involved in sensitization of B-lymphoma cells to apoptosis by rituximab see figure 2.
The AKT-pathway is another pathway that is constitutively active in B-cell lymphoma. Treatment of Ramos and Daudi cells with rituximab resulted in significant inhibition of the constitutively activated
Akt pathway (38). Inhibition the Akt pathway resulted in decreased phosphorylation of Bad. In its unphosphorylated form, Bad binds to and inactivates anti-apoptotic proteins, such as Bcl-2 and Bcl-xL, leading to its proapoptotic function. In addition, inhibition of active Akt inhibits NF-kB activity via inhibition of IκB. Treatment of tumor cells with the Akt-inhibitor LY294002 or with Akt siRNA, but not control siRNA, resulted in inhibition of Bcl-xL expression and sensitization to drug-induced apoptosis. Hence, inhibition of the Akt pathway by rituximab resulted in significant inhibition of both function and expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL. This supports the contribution of rituximab-induced inhibition of the Akt pathway in chemosensitization.

As mentioned before, recently established rituximab-resistant (RR) B-NHL cell clones appeared not to be chemosensitized by rituximab and exhibited higher drug resistance (42). RR clones responded less to growth inhibition by rituximab, complement mediated lysis, or rituximab-induced apoptosis. Both NF-κB and ERK1/2 survival pathways were described to be constitutively hyperactivated in these clones, leading to overexpression of resistant factors like Bcl-2, Bcl-xL or Mcl-1 (42). This supports the evidence that rituximab-mediated chemosensitization occurs via NF-κB and ERK 1/2 survival cascades and downregulation of anti-apoptotic factors like Bcl-2, but simultaneously presents one of the difficulties for repeated treatment with rituximab; the development of rituximab-resistance.

Next to sensitization to various chemotherapeutic drugs, Vega *et al* reported that rituximab can also induce sensitization to apoptosis induced by the death receptor Fas, via YY1-mediated increase of Fas expression and concurrent inhibition of the NF-κB signaling pathway (118). The capacity of rituximab to influence the membrane composition of B-cells by inducing the translocation of other proteins into rafts was also suggested by Jazirehi *et al* (42). They hypothesized that in wildtype B-NHL cells, rituximab induces a rapid generation of ceramide in lipid rafts, while in RR clones rituximab-induced ceramide generation is reduced . This suggests that rituximab resistance might partly be due to inadequate mobilization of the signaling molecules to lipid rafts and a crippled ceramide pathway. It is likely

that pathways that are inhibited in rituximab resistant clones are involved in chemosensitization in non-resistant cells. These findings support the idea that CD20 triggering influences the membrane composition of B-cells, thereby introducing a new mechanism by which CD20 could regulate cell behaviour and signaling.

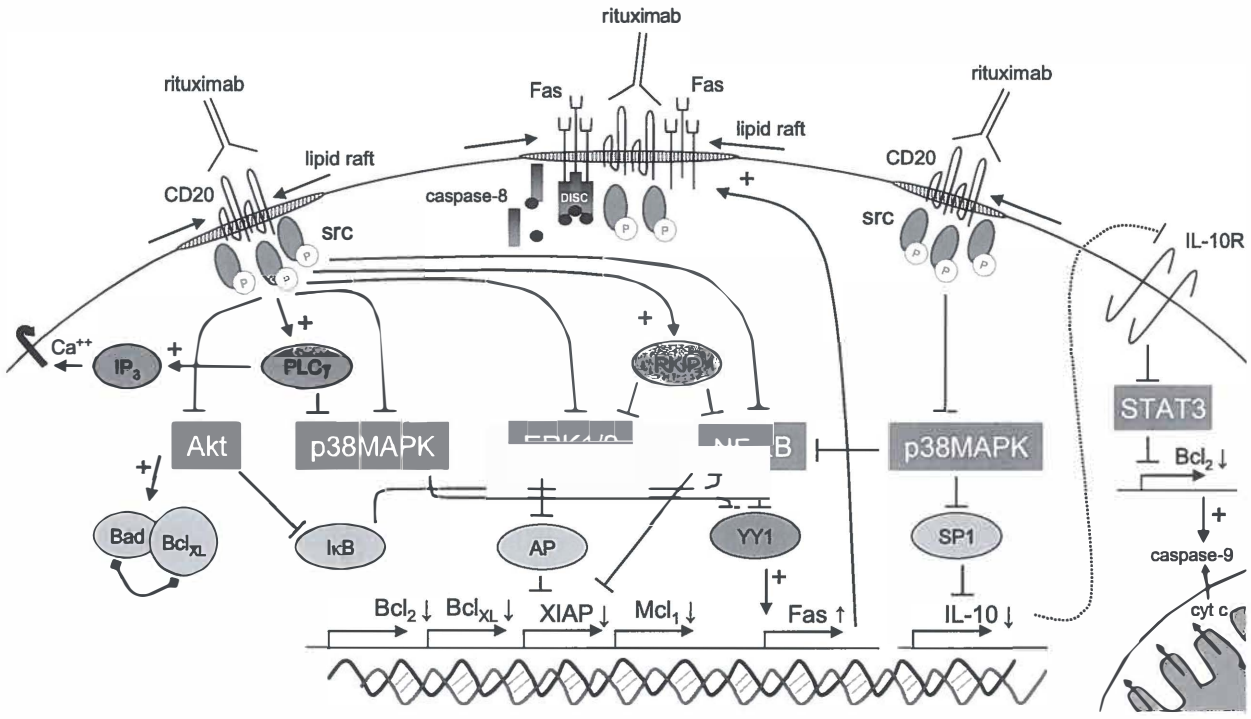

Figure 2. Chemosensitization by rituximab. Rituximab binds to CD20 thereby inducing translocation of CD20 into lipid rafts. This results in altered phosphorylation states of src-kinases. Src kinases, in turn, can regulate activity of many signaling proteins including Akt, p38MAPK, ERK1/2, NF-κB and PLCy. Akt, p38MAPK, ERK1/2, NF-κB survival pathways are constitutively active in B-cell lymphoma. CD20 stimulation results in inhibition of these constitutive activity, downregulation of IκB and decreased transcriptional activity of AP1 and SP1. These effects culmulate in decreased expression of anti-apoptotic proteins such as Bcl2, BclXL, XIAP and Mcl1. Akt inhibition also results in complex formation between Bad and Bcl_{XL}, decreasing the availability of Bcl_{XL}.Alternatively, CD20 stimulation results in calcium mobilization via PLCy activation and in activation of RKIP, resulting in downregulation of ERK1/2 and NF-κB. Inhibition of p38MAPK or NF-κB also inhibits the transcription repressor YY1 which negatively regulates Fas expression resulting in upregulation of Fas and sensitization to Fas-mediated apoptosis. Translocation of CD20 to lipid rafts is also spontaneously accompanied by clustering of Fas molecules and formation of the DISC, increasing Fas sensitization. Finally, inhibition of p38MAPK results in decreased transcriptional activity of SP1, a transcription factor responsible for IL-10 production. Downmodulation of IL-10 secretion inhibited STAT3 activity, also resulting in downregulation of Bcl2 and sensitization to chemotherapeutic drugs.

CD20-family and related proteins

The CD20 molecule is member of a relatively unknown membrane protein family, named the membrane-spanning four-domains subfamily A (MS4A-family). Other family members are the hematopoietic cell-specific protein HTm4 and the high affinity IgE receptor beta-chain (FcεRIβ) (119). Proteins of this group are highly hydrophobic, they share a similar structure and show 25-40% amino acid homology (120,121). All members of the MS4A family are likely to be components of oligomeric cell surface complexes involved in signal transduction. They are expressed in various tissues, but mostly in lymphoid tissues such as spleen, thymus and intestines (121). FcεRI consists of a ligand-binding alpha-chain and two kinds of signaling chains, the beta-chain and the disulfide-linked homodimeric gamma-chains (122). Signaling, initiated by the ligand-receptor interaction, is conveyed to the FcεRIβ subunit, which shows redistribution to lipid raft domains and functions as an amplifier of FcεRI-mediated cell activation (123,124).To date, no *in vivo* natural ligand has been described for CD20. Although CD20 signaling is extensively described upon induction by Ab binding, induction of CD20 signaling *in vivo* has never been reported to be a consequence of receptor-ligand interaction. Maybe, similar to the FcεRIβ chain, CD20 could be a subunit of a larger complex to which signals, obtained via a closely related complex partner, are conveyed and subsequently amplified by CD20.

A comparable function has been shown for a group of proteins closely related to the MS4A-family: the tetraspanins (125). Like CD20, tetraspanins are characterized by four transmembrane domains of which both the amino- and carboxy-termini are positioned at the cytoplasmic side of the membrane. Tetraspanins are abundantly expressed in cells of the immune system where they have been reported to orchestrate intermolecular interactions at the membrane (126). The extended, tetraspanin-based multimolecular complexes, known as the 'tetraspanin web' are instrumental to various crucial aspects of immune functioning requiring orchestration of close proximity protein interactions and signal transduction (127). A number of tetraspanins have been shown to associate with MHC class II molecules, facilitating immune synapse formation and antigen presentation to T-cells (128-130). CD20 is both physically and functionally coupled to HLA-DR on B-cells (131,132). Upon Ab stimulation of HLA-DR, a number of typical CD20-like cell-biological features are initiated. These include association with lipid rafts (133), changes in calcium conductance (134), and apoptosis induction (135). Comparable to CD20, HLA-DR stimulation activates Src family of tyrosine kinases with downstream activation of phospholipase Cγ (PLCγ) and regulates mitogen-activated

protein kinases (MAPKs), such as ERK and p38 MAPK (134). Type II mAbs directed to CD20 and mAbs specific for HLA-DR were shown to elicit similar peripheral relocalization of actin, homotypic adhesion and related cell death via a lysosome-dependent pathway (64). Leveille *et al* showed a direct association between HLA-DR molecules and CD20 by immunoprecipitation, and CD20 appeared to be a major component of MHC class II mediated activation of Src kinases. Indirectly, MHC class II molecules were described to contain binding sites for CD77 (globotriaosyl ceramide, a raft associated glycosphingolipid present on germinal center B-cells), suggesting a regulatory role for CD77 in antigen presentation, whilst others described a selective interplay between CD77 and CD20 (136,137). Also, CD20 appeared to directly interact with CD81, one of the members of the tetraspanin family that co-localizes with MHC class II molecules at the site of the immunological synapse and promotes lipid raft translocation of associated receptors, but is also known to regulate activation of the IgE receptor containing the MS4A-family member FcεRIβ (138-140). Recent experiments by Stein *et al* demonstrated HLA-DR mediated sensitization of B-cell lines, including rituximab resistant lines, to rituximab mediated proliferation inhibition (141). Overall, CD20 might play a role in efficient activation of the B-cell via the MHC class II/antigen presentation route.

In this thesis we show that HLA-DR and CD20 signaling follow similar kinetics and we provide evidence that CD20 and HLA-DR show functional interaction (142). We hypothesize that CD20 has a biological function in amplifying the signals of related receptor molecules like MHC class II molecules or can, like tetraspanins, facilitate immune synapse formation and receptor signaling in B-cells.

CD20 targeting treatment modalities

Many of the above described facts, theories and hypotheses concerning CD20 are used in practice to improve therapies and the clinical outcome of B-cell lymphoma patients. Next to the extensive clinical knowledge that is obtained for rituximab, either as a single agent or in combination with chemotherapeutic agents, other potential agents are investigated for their potential use in the clinic as well. Radioimmunotherapy (RIT) is one of the new modalities for treating lymphomas. The first RIT agent approved by the US FDA was yttrium-90 (^{90}Y) ibritumomab tiuxetan (Zevalin) consisting of the monoclonal IgG1 Ab ibritumomab (the murine parent of rituximab) bound to the chelator tiuxetan and linked to the beta-emitting radionuclide ^{90}Y (143). Another CD20 targeting RIT agent studied in clinical trials is Iodine-131 (^{131}I) tositumomab (Bexxar), the IgG2a murine monoclonal Ab (B1)

linked to the both beta- and gamma-emitting radionucleotide ^{131}I (144). Both RIT agents were clinically successful, obtaining ORR up to 82% and 95% for Zevalin and Bexxar respectively (145,146).

In addition to radioisotopes, cytokines have also been coupled to anti-CD20 Abs, as for example the DI-Leu16-IL2 immunocytokine (147). This engineered molecule is based on the anti-CD20 Ab Leu16 of which the V region sequences of both the H and L chain were de-immunized (modified to reduce affinity), and subsequently fused to recombinant IL2. In a SCID mouse model of disseminated, residual lymphoma it was more effective than the parental CD20 Ab or mock targeting control immunocytokines.

Extensive research has also been performed using bispecific antibodies (BsAbs) for treatment of B-cell malignancies. The dual specificity of these Abs couples immune effector cell activation to tumour cell recognition, thereby inducing MHC-unrestricted killing of tumour cells. CD3xCD19 BsAbs, targeting both the CD3ε chain of the T-cell receptor and the pan-B-cell antigen CD19, were shown to redirect and activate autologous T-cells to CD19 expressing malignant B-cells *in vitro* in the presence of monoclonal costimulating CD28 Abs (148). *In vivo* analysis in immunodeficient mice showed that CD3xCD19 BsAbs in combination with CD28 Abs inhibited growth of engrafted human B-cell lymphoma cells (149). Tested in a clinical phase I trial, CD3xCD19 BsAbs plus CD28 Abs induced only moderate side effects, but resulted in a limited local clinical response in only 22% of the patients treated (150). Additionally, binding of Abs to CD19 leads to the internalization of CD19 (151,152). Internalization of an engaged Ab is relevant when e.g. toxic substrates need to be delivered into the cell. When extracellular interactions between cells are needed for therapeutic effect, such as for CD3xCD19, internalization causes reduced availability of CD3 for T-cell targeting CD19-targeting Abs might therefore be considered as a preferred reagent for delivery of cytotoxic agents rather than for T-cell retargeting therapy (153). However, it was described that *in vivo* anti-tumour effects of unconjugated CD20 mAbs appeared superior to those of CD19 mAbs (154,155). A BsAb against the myeloid receptor for IgA (CD89; FcαR1) and CD20 in combination with myeloid growth factors showed effective killing in a broad range of B-cell lines (156). The combination of BsAb fragments ($bsFab_2$) with CD20xCD3 and CD20xCD28 specificity induced autologous T-cell activation and enhanced cytotoxicity in malignant B-cells in peripheral blood and bone marrow cultures from patients with CLL and follicular lymphoma in a more efficient manner than BsAbs targeting CD19 (157).

Another BsAb CD3xCD20 (CD20Bi) was described to enhance killing of CD20 expressing B-cell lines and circumvent complement-mediated rituximab resistance *in vitro* (158). Recently, Stanglmeier *et al* showed that a BsAb CD3xCD20 (Bi20), but not rituximab, upregulated the activation markers CD25 and CD69 on both CD4 positive and CD8 positive T cells in the presence of accessory immune cells (159). Bi20 induced a strong Th1 cytokine pattern characterized by high IFN-gamma and very low IL-4 secretion. They also studied if Bi20 could induce graft-versus-lymphoma responses in six patients with CLL or high grade NHL, in combination with (donor lymphocyte infusion) DLI or mobilized (peripheral blood stem cell transplant) PBSCT after allogeneic transplantation (160). All three CLL patients showed a transient clinical and hematological response. One patient with high grade NHL demonstrated a halt in progression for almost 4 months. The cytokine profile was characterized by transient increases of IL-6, IL-8 and IL-10. This suggested immunomodulation by CD20xCD3 BsAbs offering new immunotherapeutic options for the treatment of B-cell mediated diseases.

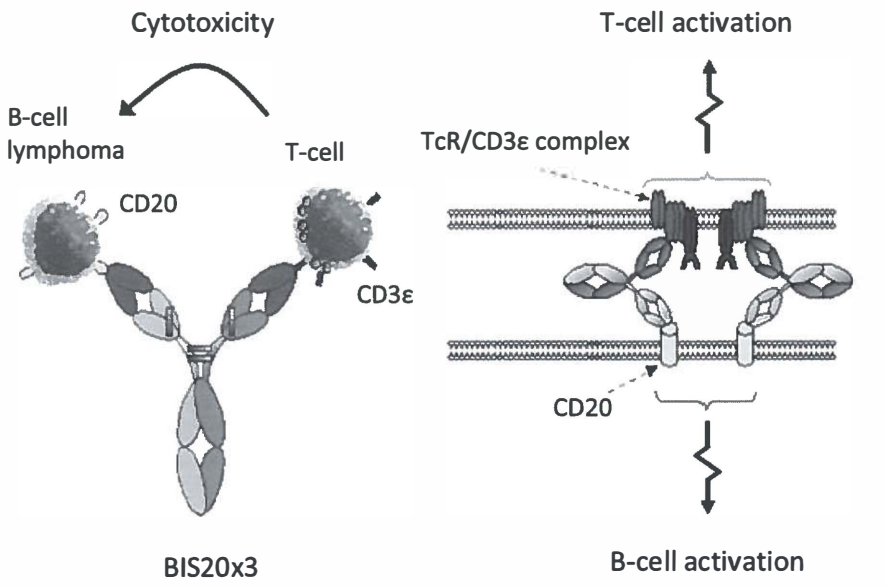

Figure 3. BIS20x3 binds to CD20 on B-cells and to CD3ε on T-cells. BIS20x3 engagement activates both B-cells and T-cells. Eventually this results in T-cell-induced cytotoxicity in B-cells.

In this thesis we describe the properties of a new CD20 and CD3 targeting BsAb (BIS20x3), developed in our laboratorium (figure 3). BIS20x3 was obtained by subcloning of quadroma cells after hybrid hydridoma fusion of the parental cell lines B-ly1 (anti-CD20) and CLB-T3/4.2B (anti-CD3ε). BIS20x3 was demonstrated to activate and retarget cytotoxic T-cells to target B-cells. We also showed that targeting CD20 by BIS20x3 induces B-cell activation, increasing its costimulatory capacity towards CD3 positive effector T-cells. Thus, CD20 appears not to be just a passive target, but can actively contribute to the effectiveness of BIS20x3 therapy, either via costimulation of the effector T-cells or by sensitization of the target cells to T-cell mediated apoptosis. The above described studies provide a multitude of indications that the use of CD20 as a target in immunotherapy, either as a monoclonal Ab, in RIT, as immunocytokine or as BsAb appears to be a very promising approach to improve lymphoma or auto-immune disease directed therapy.

Aims of the thesis

The aim of this thesis is to characterize the immunobiological features of CD20 in relation to its effectiveness as a target for therapy of lymphomas. As part of this thesis, a new therapeutic modality, the BsAb BIS20x3, is described in particular detail. This BsAb, activating and retargeting cytotoxic T-cells towards CD20 expressing B-cells is characterized for its binding capacities and cytotoxic potential at an *in vitro* level in **chapter 2**. In **chapter 3** the role of B-cell mediated T-cell costimulation in the efficacy of T-cell retargeting by BIS20x3 is studied. In this chapter evidence is given to support the hypothesis that via CD20 signaling, B-cells become activated in such a way that they increase their costimulatory capacity towards potential cytotoxic T-cells, thereby participating in their own demise. In **chapter 4**, we focus more in depth on the signaling capacities of CD20 on malignant B-cells. We show that rituximab can induce apoptosis via a death receptor/caspase 8 dependent pathway. By its capacity to induce ligand independent translocation of the death receptor Fas into multimolecular clusters, rituximab renders malignant B-cells more prone to Fas-mediated apoptotis induction. In **chapter 5** the attention is directed to the possible functional role of CD20 on the membrane of B-cells. Evidence is provided for a shared signaling pathway for CD20 and HLA-DR. Additionally, it is shown that a functional MHC class II complex, containing HLA-DR, is necessary for CD20 to pass on its apoptotic signal upon Ab crosslinking. Furthermore, HLA-DR stimulation appears to create a suitable environment for CD20 to translocate to raft domains, without CD20 Ab stimulation. We hypothesize that CD20 might have a biological function in amplifying the signals of related receptor molecules like HLA-DR or can, like tetraspanins, facilitate immune synapse formation and receptor signaling in B-cells.

We suggest major benefits from BIS20x3 in T-cell mediated lymphoma treatment considering that the BsAb can benefit from specific CD20-related features. BIS20x3 can effectively activate and retarget cytotoxic T-cells to malignant B-cells *in vitro*. BIS20x3 does not need additional costimulation to induce optimal T-cell activation. Additionally, CD20 stimulation renders B-cells more sensitive to Fas induced apoptosis, one of the major cytotoxic effector mechanisms of activated T-cells. These facts, combined with the finding that CD20 signaling shares great homology with and even participates in HLA-DR signaling, encouraged us to further explore the possibilities of the BsAb BIS20x3 in an *in vivo* setting. In **chapter 6** the effectiveness of BIS20x3 in a SCID/NOD mice model is described, showing the kinetics of the Ab in an *in vivo* situation, demonstrating efficient, CD20 specific, T-

cell activation and inhibition of tumour growth within this established model for human acute lymphatic leukemia (ALL) engraftment in a murine host.

In conclusion, in this thesis we demonstrate BIS20x3 to be a promising approach to improve CD20-directed B-cell therapy. This is substantiated by providing new insights concerning CD20 signaling and its biological function in B-cells.

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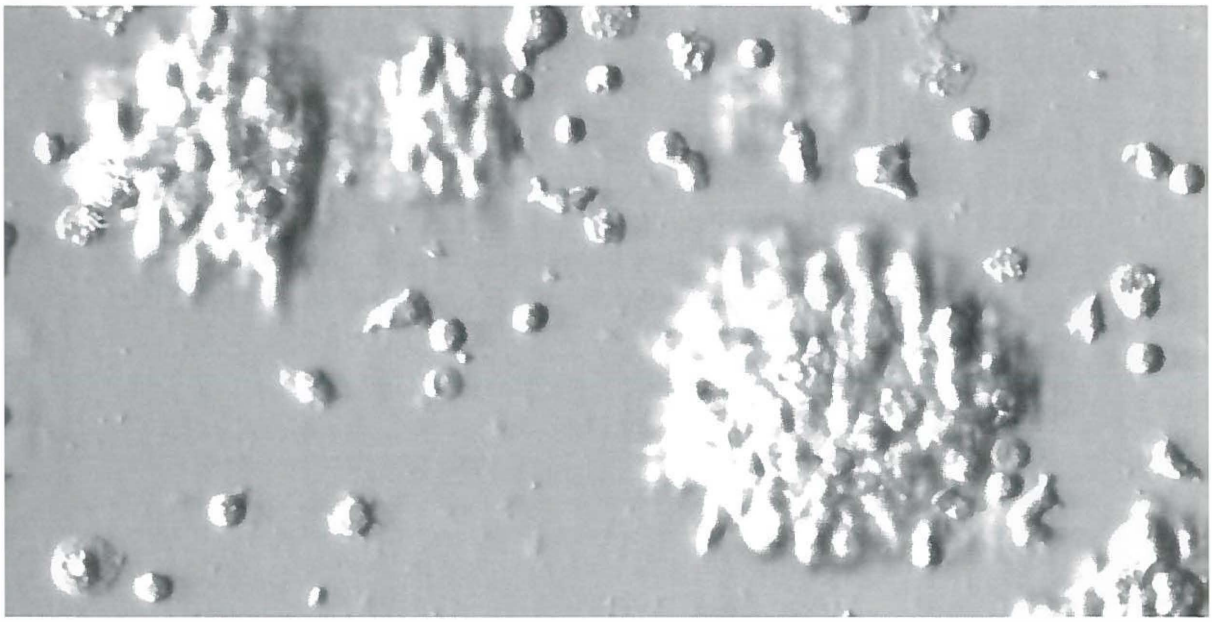
Chapter 2



Characterization of BIS20x3, a bispecific antibody activating and retargeting T-cells to CD20-positive B-cells.

British Journal of Cancer (2001), 84: 1115-1121.

Abstract

This paper describes a bispecific antibody, which was called BIS20x3. It retargets CD3ε-positive cells (T-cells) to CD20-positive cells and was obtained by hybrid-hybridoma fusion. BIS20x3 could be isolated readily from quadroma culture supernatant and retained all the signaling characteristics associated with both of its chains. Crosslinking of BIS20x3 on Ramos cells leads to DNA fragmentation percentages similar to those obtained after rituximab crosslinking. Crosslinking of BIS20x3 on T-cells using crosslinking F(ab')2-fragments induced T-cell activation. Indirect crosslinking of T-cell bound BIS20x3 via Ramos cells hyper-activated the T-cells. Furthermore, it was demonstrated that BIS20x3 effectively retargets T-cells to B-cells, leading to high B-cell cytotoxicity. The results presented in this paper show that BIS20x3 is fully functional in retargeting T-cells to B-cells and suggest that B-cell lymphomas may represent ideal targets for T-cell retargeting bispecific antibodies, because the retargeted T-cell is maximally stimulated in the presence of B-cells. Additionally, since B-cells may upregulate CD95/Fas expression upon binding of CD20-directed antibodies, B-cells will become even more sensitive for T-cell mediated killing via CD95L/FasL, and therefore supports the intention to use T-cell retargeting bispecific antibodies recognizing CD20 on B-cell malignancies as a treatment modality for these diseases.

Introduction

The concept of using Abs as 'magic bullets' for cancer therapy has shown a remarkable revival. Recently highly encouraging results have been reported with Abs targeting EGP-2/EpCAM on carcinomas (Edrecolomab; (1)), HER-2/neu on breast cancers (Trastuzumab; (2)) and CD20 on lymphomas (rituximab; (3)). Impressive response rates are obtained with these Abs alone or in combination with additional chemo-therapeutic and/or radiotherapeutic regimens, without showing significant cytotoxicity. The most promising of these Abs are those targeting the B-cell differentiation marker CD20. These Abs have been used for treatment of non-Hodgkin lymphoma (NHL) patients, as more than 90% of all non-Hodgkin lymphomas are positive for CD20. After rituximab treatment 50% of patients with advanced, indolent NHL showed partial or complete responses (3). Because CD20 is expressed on B-cells during a number of stages of B-cell development, but is absent from B-cell progenitor cells and mature plasma cells (4), anti-CD20 Abs do compromise the patient's immune system only to a limited extent.

The effectivity of rituximab can probably be explained by its supposed dual effector function. First it is known that Abs retarget the immune system to Ab-coated cells via their Fc-tail, leading ultimately to death of the cell by complement mediated lysis and/or antibody-dependent cellular cytotoxicity. Secondly, it has been shown that binding of Abs to CD20 can modulate B-cell proliferation and/or antibody-dependent cellular cytotoxicity and that crosslinking of anti-CD20 Abs (e.g. by Fc-receptor positive cells) on B-cells can lead to apoptosis of the target cell (5,6). The latter effects are thought to contribute significantly to the efficiency of treatment with this 'signaling antibody' (2). Although the results obtained with rituximab are very promising it is important to note that anti-CD20 treatment is not curative and that treated patients may relapse ultimately. In order to improve upon the efficiency of anti-CD20 Ab treatment several strategies have been investigated. One approach is the labeling of the Abs with radioisotopes to induce local irradiation. In another approach toxins are coupled to the Abs and by this means delivered to tumour cells. Also combination therapy regimens consisting of rituximab and chemotherapy have been tested in clinical trials (3). Although these approaches are indeed more successful (higher response rates were achieved) increased toxicity to normal tissues may be expected.

In the past we have focused on the development of BsAbs which are designed to retarget T-cells to tumour (associated) antigens. In the setting of NHL it might be expected that BsAb-mediated retargeting of T-cells to CD20-positive tumour cells

will induce apoptosis more efficiently than CD20 crosslinking alone, because T-cells are equipped to optimally induce apoptosis in target cells. In this paper we describe the production and characterization of the BsAb BIS20x3 via hybrid hybridoma techniques. BIS20x3 recognizes CD20 with one chain and CD3ε (on T-cells) with the other. A highly efficient activation of T-cells was observed after application of BIS20x3 and subsequent indirect crosslinking via CD20-positive cells. The results presented in this paper demonstrate that BIS20x3 is an attractive candidate for being tested as a new treatment modality for CD20-positive malignancies. B-cell malignancies may be ideal targets for T-cell retargeting BsAbs because B-cells may efficiently costimulate the effector T-cells.

Materials and methods

Culture media and other reagents.

RPMI 1640 medium (containing 25 mM Hepes and L-glutamin) and 100x HT-supplement were obtained from Bio-Whittaker (Verviers, Belgium) and FCS from Bodinco BV (Alkmaar, The Netherlands). Culturing 'additives' consist of 1 mM sodium pyruvate (Bio-Whittaker), 2 mM L-glutamin (Bio-Whittaker), 0.5 mM β-mercaptoethanol, 0.1 mg/ml gentamycin sulphate (Bio-Whittaker), and fungizone (Amfotericine-B, Bristol-Myers Squibb, Woerden, The Netherlands). Hygromycin was purchased from Roche Molecular Biochemicals (Almere, The Netherlands), PFHM-II (protein-free hybridoma medium) containing glutamax-I from Gibco BRL-Life Technologies (Breda, The Netherlands), calcein-AM from Molecular Probes (Leiden, The Netherlands), protein-A (PROSEP®-A) from Bioprocessing Ltd (ImmunoSource, Zoersel-Halle, Belgium), and Triton X-100, propidium iodide (PI) and RNase-A from Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands). Lymfoprep was obtained from Nycomed Pharma AS (Oslo, Norway) and serum- and phenolred-free medium (X-VIVO™) from Bio-Whittaker.

Hybridomas, quadroma and cell lines.

Hybridoma and quadroma cell lines were cultured in RPMI 1640 medium containing 15% FCS, 15% ESG (conditioned ES-1-culture medium, IQ-Products, Groningen, The Netherlands), additives (see above), and HT-supplement. All other cell lines were cultured in essentially the same medium, however without conditioned ES-1 culture medium and HT supplement. All cells were cultured at 37°C, in a humidified atmosphere containing 5% CO_2. The EBV-immortalized human B-cell line JY was obtained from Dr L Bakker (Dept. Tumor Immunology, Nijmegen, the Netherlands). CTLL/CD3ε is a mouse T-cell line transfected with cDNA encoding human CD3ε (7). Jurkat-AM, which was obtained from Dr P Schrier (University Hospital Leiden, Leiden, The Netherlands), was cultured in the presence of 0.5 mg/ml hygromycin in order to select for NFAT-luc (the reporter gene luciferase under control of the NFAT-promoter) positive cells.

Commercial antibodies.

FITC-, TRITC- or PE-labelled anti-IgG1- or anti-IgG2b-Abs were obtained from Becton Dickinson (Erembodegem-Aalst, Belgium) or from IQ-Products (Groningen, the Netherlands). Goat-anti-mouse F(ab')2-fragments (GaM-F(ab')2) and goat-anti-human F(ab')2-fragments (GaH-F(ab')2) used for crosslinking of respectively mouse-and human anti-CD20 Abs were obtained from Jackson Laboratories (West Grove, PA, USA) as well as anti-human IgM-Ab. The anti-CD3-Ab B-B11 was obtained from IQ-Products.

Hybrid hybridoma fusion, subcloning of quadroma cells and characterization of the antibodies produced by the sub-clones

A quadroma cell line was derived after fusion of the hybridoma cell lines B-ly1 (anti-CD20, IgG1; (8)) and CLB-T3/4.2B (anti-CD3ε, IgG2b; (9,10)). Fusion was performed by standard hybrid hybridoma fusion techniques as described previously (11). Briefly, a HGPRT-negative anti-CD3ε hybridoma cell line was selected by culturing it in 8-azaguanine containing medium. This hybridoma cell line, unable to grow in hypoxantine, aminopterine- and thymidine-(HAT) containing medium, was made neomycin resistant by retroviral transfection. This HAT-sensitive, neomycin-resistant cell line was then fused with the HAT-resistant, neomycin-sensitive hybridoma cell line B-ly1. Standard polyethyleneglycol (PEG) fusion procedures were used. Quadroma cells were subcloned in 96-well plates and clones producing both IgG1- and IgG2b-chains were identified by detection of fluorescence after labeling the quadroma cells with FITC- or TRITC-labeled anti-IgG1- or anti- IgG2b-Abs. One subclone was selected. In order to validate CD20- and CD3ε-binding capacity of the Abs produced by this clone, culture supernatant was harvested and applied to CD20-positive JY cells or CD3ε-positive CTLL/CD3ε cells.

Partial purification of BIS20x3.

Twice weekly Ab-producing cells were seeded in PFHM-II medium. Supernatant was harvested when the number of dead cells exceeded 30%. Approximately 500 ml supernatant was collected and concentrated 10 times using the ProVario-3FP concentrator (Filtron Technology BV, Terheyden, the Netherlands). Antibody purification was performed by FPLC (Pharmacia, Uppsala, Sweden). Concentrated supernatant was diluted two-fold in 0.1 M K-phosphate buffer pH 8.2 (equilibration buffer) and loaded onto a protein-A column. Antibodies were eluted using a 0.1 M Na-citrate buffer pH-gradient, decreasing from pH 6 to pH 2. Supernatants of the parental hybridomas were subjected to the same procedure in order to determine at which pH the parental Abs elute from the column. During elution protein peaks were detected spectrophotometrically (OD280) and 0.5 ml fractions were collected. In each fraction the pH was measured and neutralized by adding increasing volumes of a 1 M K-phosphatesolution (pH 9.4). Each fraction was analyzed for the presence of IgG1- and/or IgG2b-isotype Ab chains by FACS analysis (Epics Elite, Coulter, Hialeah, USA) using the Abs described above. The presence of the various Ab species was confirmed by SDS/PAGE gel electrophoresis and subsequent Western blotting.

Detection of DNA fragmentation after crosslinking of anti-CD20 antibodies on CD20-positive cells.

To induce apoptosis Ramos cells were harvested and incubated for 30 min with the different anti-CD20 Ab concentrations (see Results) at 37°C. The cells were washed to remove unbound Ab and plated at a concentration of 0.5-1x10^6 per well (6 well plates, 2.5 ml per well). Crosslinking was achieved by adding crosslinking F(ab')2-fragments. The next day the Nicoletti assay was performed to determine the percentage (%) apoptotic cells in the population. This assay is based on detection of a hypoploid DNA peak by FACS analysis (12). After 16 hours apoptosis induction the cells were washed in PBS containing 1% BSA and resuspended in 0.2% Na-citrate, 0.2% Triton X-100, 0.2 mg/ml RNase-A. DNA was stained by adding 100 µg/ml PI. PI fluorescence was measured by FACS.

Detection of T-cell activation after CD3ε-crosslinking.

A TcR-beta chain negative Jurkat cell line harbouring an NFAT-luciferase reporter gene named Jurkat-AM, was retrovirally transduced with genes encoding the alpha and beta TcR chains from an HLA A2-restricted anti-MAGE-3 T-cell clone (13). The resulting subline is called Jurkat-AM/T. Transduction restored CD3ε-expression on the surface of Jurkat-AM/T as was determined by FACS (not shown). After binding of anti-CD3ε-Abs to Jurkat-AM/T, the NFAT-promoter becomes activated which results in luciferase expression. Approximately 1.5x10^6 cells per well (24 well plates) were activated during the period chosen. When necessary 0.5x10^6 Ramos cells were added. The cells were harvested and washed once. Pellets were frozen at -20°C for at least 15 min to facilitate lysis which was performed in lysis buffer supplied by the manufacturer of the Luciferase Assay System (Promega, Leiden, The Netherlands). Luciferase activity was detected using the same kit. Luminescence was detected on the Anthos Lucy Microplate Luminometer and Photometer (Labtech International, Ringmer, UK).

Cytotoxicity assays.

We have used the calcein-release assay (14) as an alternative for the ^{51}Cr-release assay to demonstrate that BIS20x3 retargets T-cells to CD20-positive cells. PBLs were isolated from heparin-blood using lymfoprep according to protocols described by the manufacturer. T-cells were activated and expanded by incubation for three days in RPMI 1640 medium containing 15% normal human poolserum and 5% WT-32 (anti-CD3ε-hybridoma) supernatant (15), followed by two days culturing in RPMI 1640 medium containing 15% normal human poolserum and 100 IU/ml IL-2. JY-cells were used as the CD20-positive target cells. JY cells were isolated, washed and resuspended in X-VIVO-medium at a concentration of 2 x 10^6 cells/ml. Calcein-AM was added to the cells in a concentration of 8 mM for 40 min, after

which extracellular calcein-AM was removed by washing. Calcein-AM is the acetoxy-methylester of calcein which diffuses passively across cell membranes. In the cytosol calcein-AM is converted into the fluorochrome calcein by intracellular esterases. Calcein is retained in cells with intact membranes due to its polar nature. We applied $2.0x10^4$ labeled JY target cells per well to 96-well plates, and varied the number of T-cells (also washed and resuspended in X-VIVO-medium) to obtain varying effector-target cell ratios. After two hours incubation in the absence or presence of different Abs, the cells in the plates were pelleted and the supernatant was transferred into a new 96-well plate. Calcein fluorescence in the supernatant was determined on the Bio-Tek FL500 fluorescence plate reader (BioTek® Instruments Inc, Burlington, USA; excitation at 485 nm, emission at 530 nm). The % cytotoxicity was calculated using the equation: $(F_{sample} - F_{spontaneous\ release}) / (F_{total\ lysis} - F_{spontaneous\ release}) \times 100\%$ = % cytotoxicity. Total lysis values were obtained by addition of 0.5% Triton X-100 to labeled JY cells. The ^{51}Cr-release assay was performed as described previously (16).

Results

Hybrid hybridoma fusion.

The quadroma cell line producing BIS20x3 was made by fusion of B-ly1 and CLB-T3/4.2B. The quadroma was screened for expression of both parental Ab chains and cultured after subcloning twice. A clone displaying more than 95% double positive cells was selected as the 'producer' cell line. Additionally, culture supernatant was applied to CD20- or CD3ε-positive cell lines (JY or CTLL/CD3ε respectively). The co-presence of Ab chain subclasses which do not bind by themselves on the target cells (anti-CD20-chains (of IgG1-subclass) on CTLL/CD3ε cells or anti-CD3ε-chains (of IgG2b-subclass) on JY-cells) suggested that the BsAb was formed and confirmed the antigen-binding capacity of both chains (results not shown).

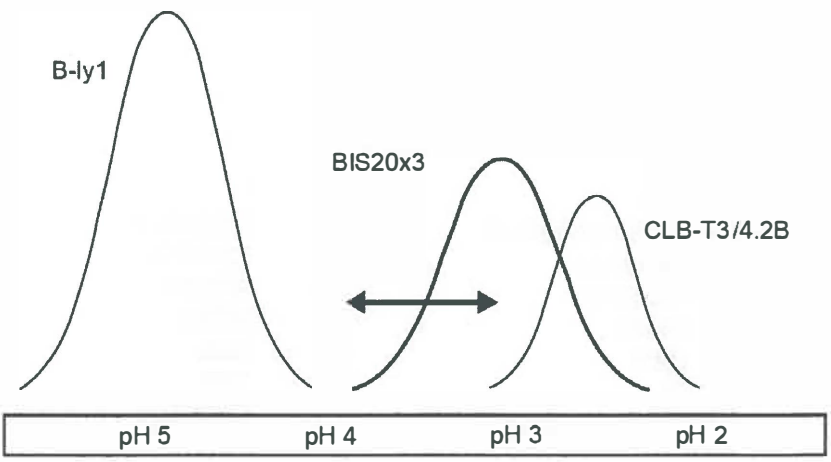


Figure 1. Schematic representation of the pH elution profiles of both parental antibodies (B-ly1 and CLB-T3/4.2b) and the bispecific antibody (BIS20x3). The double arrow indicates the fractions used for the experiments described in this paper.

Isolation and partial purification of the bispecific antibody (BIS20x3).

Culture supernatants from B-ly1 (anti-CD20), CLB-T3/4.2B (anti-CD3ε) or the quadroma culture were isolated and concentrated. First, samples of the concentrated parental hybridoma supernatants were mixed and loaded onto a protein-A FPLC column to elucidate the pH-elution profile of both parental Abs. Using FACS- and Western blot analysis it was shown that the parental anti-CD20 Ab B-ly1 leaves the column between pH 5.5 and pH 4 and that the anti-CD3ε-Ab elutes

between pH 3 and 2 (peak at pH 2.5). In Fig 1 the elution profiles are depicted. The results suggest that the parental Abs can be separated from each other. After loading quadroma supernatant and subsequent Ab elution using the same pH gradient, it was demonstrated that the BsAb leaves the column at a pH value lower than pH 3.8 indicating that the BsAb, which was called BIS20x3, could be isolated without 'contaminating' B-ly1. However, the BIS20x3 peak overlaps partially with the anti-CD3ε-peak. Therefore it was decided to use only the fractions isolated from the first half of the BIS20x3-peak for the experiments described below (more than 90% pure regarding BIS20x3-content). To check the functionality of the antigen-binding capacity of both arms of the BsAb, assays were subsequently performed which detect the intracellular signals which are relayed after binding of Abs to CD20 on B-cells or CD3ε on T-cells, respectively.

Detection of DNA hypoploidy after crosslinking of anti-CD20 Abs on Ramos cells.
It was reported that binding of anti-CD20 Abs to CD20 and subsequent crosslinking of these Abs leads to apoptosis induction in Ramos cells (5).

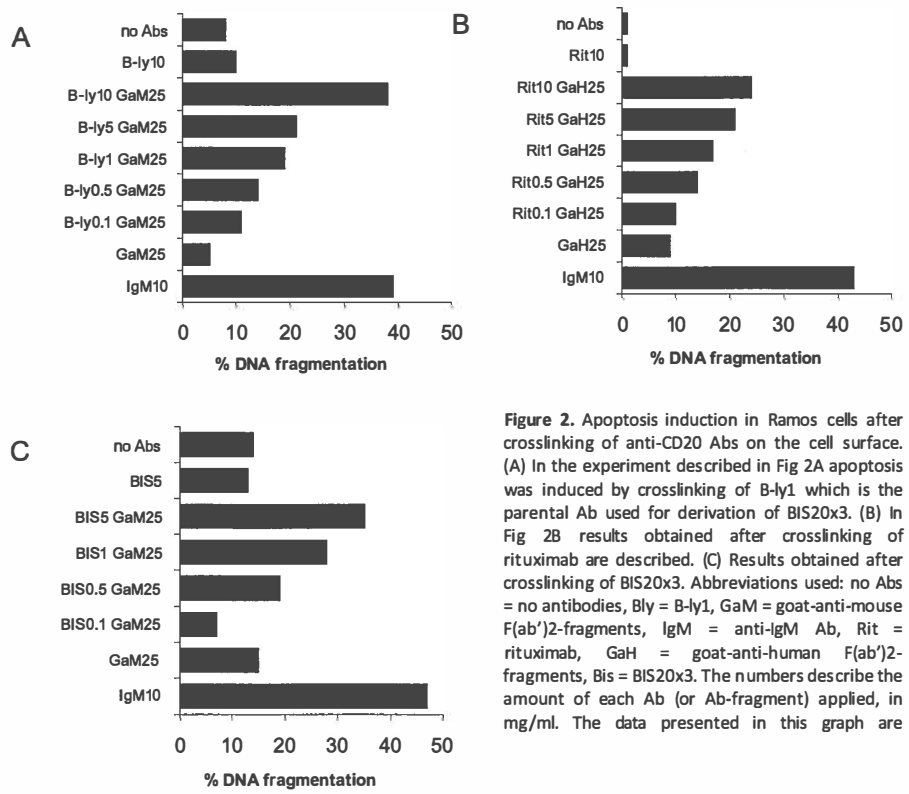


Figure 2. Apoptosis induction in Ramos cells after crosslinking of anti-CD20 Abs on the cell surface. (A) In the experiment described in Fig 2A apoptosis was induced by crosslinking of B-ly1 which is the parental Ab used for derivation of BIS20x3. (B) In Fig 2B results obtained after crosslinking of rituximab are described. (C) Results obtained after crosslinking of BIS20x3. Abbreviations used: no Abs = no antibodies, Bly = B-ly1, GaM = goat-anti-mouse F(ab')2-fragments, IgM = anti-IgM Ab, Rit = rituximab, GaH = goat-anti-human F(ab')2-fragments, Bis = BIS20x3. The numbers describe the amount of each Ab (or Ab-fragment) applied, in mg/ml. The data presented in this graph are

From Fig 2A and 2B it can be concluded that B-ly1 crosslinking leads to similar DNA-fragmentation percentages as when rituximab is crosslinked. A dosage of only 0.1-0.5 mg/ml B-ly1 or rituximab is enough to detect apoptotic cells after crosslinking during 16 hours. Also shown is that addition of anti-CD20 Ab alone or crosslinking Ab alone does not affect the Ramos cells. As a control anti-IgM Ab was applied, resulting in high percentages of DNA fragmentation. Also crosslinking of BIS20x3 leads to DNA fragmentation in Ramos cells (Fig 2C), becoming evident at a BIS20x3 concentration of 0.5 mg/ml. The levels of DNA fragmentation appear to be higher than achieved in Fig 2A and 2B, however, it has to be remarked that the untreated control shows already a relatively high apoptotic fraction in this experiment. The results presented here demonstrate that the anti-CD20 chain of BIS20x3 is functionally active in inducing apoptosis upon crosslinking.

T-cell signaling induced by binding of BIS20x3 to Jurkat-AM/T-cells.

To demonstrate the functional capacity of the anti-CD3ε-chain of CD20x3 to activate T-cells, a CD3ε signaling assay was used which was described in *Materials and Methods*. Fig 3A shows results obtained after binding of BIS20x3 to CD3ε expressed on the surface of Jurkat-AM/T-cells, and subsequent crosslinking of BIS20x3 by goat-anti-mouse F(ab')2-fragments. Crosslinking of increasing amounts of BIS20x3 results in increasing levels of T-cell stimulation. As a positive control T-cells were stimulated with WT-32 culture supernatant and as a negative control no Ab was added.

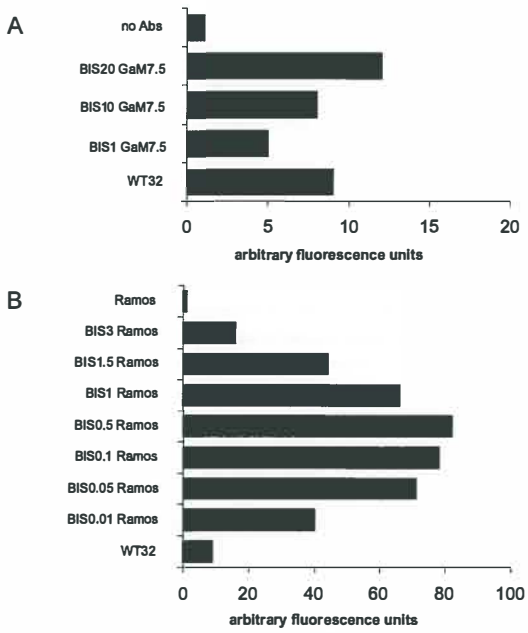


Figure 3. Luciferase activity measured in extracts of Jurkat-AM/T-cells after different treatments. (A) In Fig 3A it is shown that crosslinking of increasing amounts of BIS20x3 (BIS) using goat-anti-mouse F(ab')2-fragments (GaM) leads to increasing T-cell activation. As a negative control no Abs were added and as a positive control 100 ml WT-32 supernatant (WT32) was applied. (B) In Fig 3B the remarkable results obtained after indirect crosslinking via Ramos cells are presented. Crosslinking of varying amounts (the numbers represent mg/ml) of BIS20x3 on the surface of Jurkat-AM/T-cells via Ramos cells, leads to a dramatic increase in T-cell activation when compared with the results obtained after crosslinking using goat-anti-mouse F(ab')2-fragments. At higher levels of BIS20x3 the amount of BsAb saturates the available binding sites on both T- and B-cells which prevents crosslinking and results in a decrease in T-cell activation.

Because we also expected indirect CD3ε-crosslinking to occur (and therefore T-cell activation) when Ramos cells were added to BIS20x3-coated Jurkat-AM/T-cells, we performed the experiment summarized in Fig 3B. In this graph it is clearly shown that the T-cells are much more efficiently stimulated when BIS20x3 is crosslinked via Ramos cells than when the BsAb is crosslinked with goat-anti-mouse F(ab')2 fragments (Figure 3A), what may be caused by a costimulatory capacity of the Ramos cells. The results presented in Figure 3 demonstrate the functional binding of BIS20x3 to CD3ε.

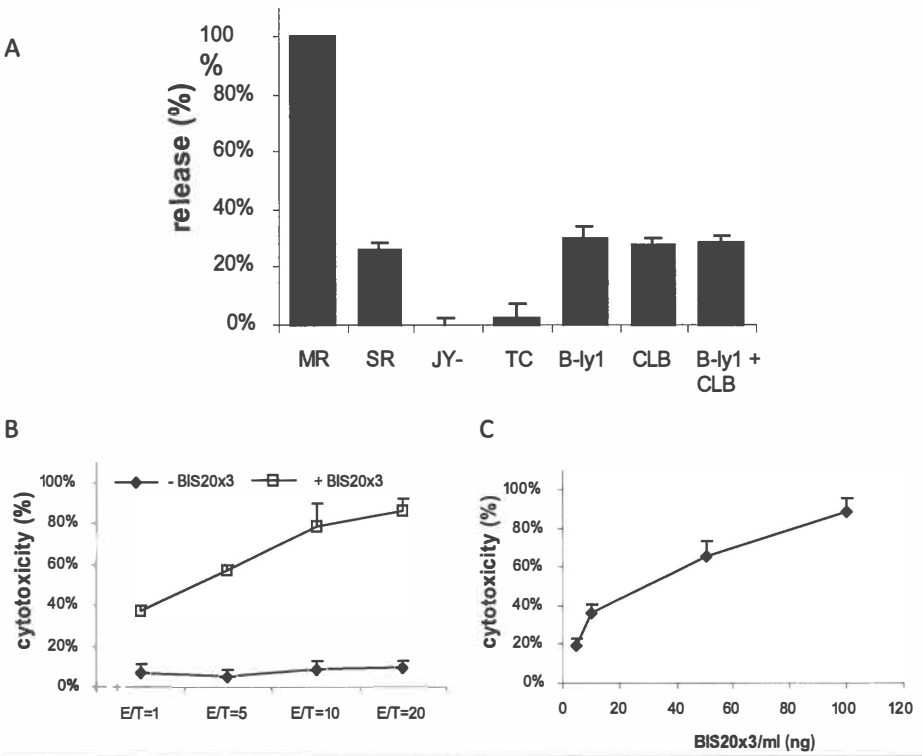


Figure 4. BIS20x3-mediated cytotoxicity in calcein-release assay. (A) In Fig 4A the calcein-release assay-controls are presented. The maximal release value (obtained from Triton-treated cells) was set to 100% and the following bars show the %-release related to this value. Shown are respectively: MR = maximal release, SR = spontaneous release, JY - = background fluorescence of medium in which unlabelled JY cells were present, TC = background fluorescence of medium in which unlabeled T-cells were present, B-ly1 = effect of 0.5 mg/ml parental Ab B-ly1 on labeled JY cells, CLB = effect of 0.5 mg/ml of other parental Ab CLB-T3/4.2B (0.5 mg/ml), and B-ly1 + CLB = addition of 0.5 mg/ml B-ly1 + 0.5 mg/ml CLB-T3/4.2B. The last 3 bars represent results obtained after adding the described Abs to T-and B-cells at an E/T ratio of 20. (B) Representative results of the calcein-release assay. Cytotoxicity percentages were calculated by implementing the formula described in the materials and methods section. BIS20x3 (0.5 mg/ml) was used to retarget PBL-derived T-cells to CD20-positive JY cells at different E/T ratios. The results of Fig 4A and 4B were obtained in the same experiment. (C) Figure 4C shows that the cytotoxicity is BIS20x3 concentration dependent (E/T-ratio of 20; concentration 5, 10, 50 and 100 ng/ml BIS20x3). The data presented in this figure could be reproduced in similar experiments.

BIS20x3 retargets PBL derived T-cells to CD20-positive JY cells.

In Figure 4 representative results obtained with the calcein release assay are presented. In Fig 4A it is shown that when using the JY cell line, spontaneous release values were observed of approximately 25%. Neither the medium taken from unlabeled JY cell cultures nor medium from unlabeled T-cell cultures displayed background fluorescence. Addition of 1 mg/ml B-ly1, CLBT3/4.2B or a combination of both parental Abs to wells containing T- and B-cells at an E/T-ratio of 20 had no effect. In Fig 4B a representative CTL-assay is described. It is shown that BIS20x3 retargets PBL-derived, pre-activated T-cells to JY cells. With increasing E/T cell ratios increased cytotoxicity percentages were observed. These results could be reproduced readily indicating the good reproducibility of this non-radioactive cytotoxicity assay. Additionally, the results obtained by using the calcein release assay were essentially similar to results obtained by ^{51}Cr-release (not shown). In none of the performed assays, rituximab was able to cause B-cell lysis in the calcein-release assay as it was performed here (essentially the results were similar to those obtained for B-ly1 described in Figure 4A). Finally, Fige 4C shows that the retargeting of T-cells to CD20 positive cells is BIS20x3-concentration-dependent and that cytotoxicity can be detected at BIS20x3 concentrations which are considerably lower (factor 50) than the anti-CD20 Ab concentrations needed to detect DNA fragmentation (Fig 2).

Discussion

The anti-CD20 Ab rituximab is concerning its ability to induce tumour response rates the most effective anti-cancer Ab in the clinic at present. Its success is most probably due to a diversity of effects which are induced after binding to CD20. First, it has been shown that rituximab can kill via complement mediated lysis. Very recently the C1q binding site on rituximab has been identified (17). The second mechanism by which rituximab is thought to elicit target cell death is via Ab-dependent cellular cytotoxicity. Although both mechanisms are potent cell death activating routes it is thought that the efficacy of rituximab is at least partially due to additional mechanisms which are directly triggered by CD20 upon binding of the Ab. The function of CD20 as a signaling molecule has been investigated by several groups. It was demonstrated that upon binding of anti-CD20 Abs 95% of all CD20 molecules present on the cell move to lipid rafts in the cell membrane which are known to be important in cellular signaling (18). It is thought that CD20 signals indirectly via tyrosine kinases which are known to be present in these rafts and which were shown to interact with CD20 (19) or directly by functioning as a calcium-channel (20,21). Recently it was demonstrated that crosslinking of anti-CD20 Abs on the surface of B-cells using F(ab')2-fragments or Fc-receptor positive cells induces apoptosis in B-cells (5). The same group confirmed that protein tyrosine kinases, increased intracellular calcium concentrations, and caspases are involved (6). In the latter paper it was also shown that CD95 (Fas) becomes up-regulated after crosslinking of anti-CD20 Abs on B-cells, but that the Fas-FasL route is not important in the in vitro setting. However, this may have important consequences for the in vivo setting. Increased Fas expression could make rituximab treated B-cells more sensitive for FasL produced by other cells, especially by T-cells (22). This is an important notion, especially in the context of T-cell retargeting e.g. using the BsAb described in this paper.

Although several effector mechanisms seem to be triggered by rituximab, 50% of NHL-patients do not respond. Recently, also patients with CD20-positive post-transplantation lymphoproliferative disorder (PTLD) were treated with rituximab and also in this group 30% of the patients did not respond (23). Various escape mechanisms which cause this non-responsiveness can be envisaged (e.g. a relative tumour cell-resistance to apoptosis induction). We hypothesize that the efficacy of treatment will increase when the delivered apoptotic signal can be strengthened and we think that this can be achieved by retargeting T-cells (professional killers) to CD20-positive cells using the BsAb BIS20x3 described in this paper.

To evaluate BIS20x3 it was investigated whether the purified product was able to induce the signals which are known to be relayed by each of its parental Abs. Using the Burkitt's lymphoma cell line Ramos as a model system in analogy to experiments performed by Shan *et al* (5) we were able to demonstrate that apoptosis was induced after crosslinking of the parental anti-CD20 Ab B-ly1, rituximab or BIS20x3. DNA fragmentation was detectable after overnight incubation using Ab concentrations of 0.1-0.5 mg/ml, which is considerably lower than the concentrations used by Shan *et al* (10 mg/ml). In the experiment depicted in Fig 2C it may seem that BIS20x3 crosslinking results in more efficient apoptosis induction (35% at a concentration of 5 mg/ml) than after crosslinking of B-ly1 or rituximab (approximately 21% at a concentration of 5 mg/ml). However, it has to be pointed out that background apoptosis was rather high in this experiment compared to the experiments summarized in Fig 2A and 2B, which obviously influences the results. BIS20x3 induces similar percentages of apoptotic cells as B-ly1 or rituximab, although BIS20x3 contains only one CD20-binding site while B-ly1 and rituximab contain two. It is likely that at the used B-ly1 and rituximab concentrations these two Abs bind monovalently. If rituximab is crosslinked in vivo by Fc-receptor positive cells as was suggested by Shan, and if this phenomenon contributes significantly to rituximab efficacy, our results imply that T-cell retargeting anti-CD20 BsAbs may be at least as effective in apoptosis induction when bound to B-cells as rituximab.

The other chain of BIS20x3 targets CD3ε on T-cells. Binding of Abs to CD3ε activates T-cells. We were able to demonstrate T-cell activation upon crosslinking of BIS20x3 on its surface using goat-anti-mouse F(ab')2-fragments (Fig 3A). To obtain these results, relatively high BIS20x3-concentrations have to be used (compare the amounts used in Fig 3A with the amounts used in to obtain the results presented in Fig 3B). T-cell activation could be enhanced dramatically by using Ramos cells as indirect crosslinkers instead of the F(ab')2-fragments (Fig 3B). The enhanced T-cell activation found in this situation is most probably caused by costimulatory signals given by the Ramos cells. These results suggest that B-cell malignancies may be ideal targets for T-cell retargeting BsAb-based therapies because the retargeted T-cells will be activated most effectively (24). This finding also suggests that no additional costimuli need to be given, which was suggested when T-cells are retargeted to other types of malignant target cells (25). The results in Fig 3B also suggest that for optimal effects the BsAb concentration needs to be titrated, which may become an issue when dose-escalating clinical studies are planned. If the concentration of BsAb gets too high, the binding sites on T- and B-cells may become saturated without crosslinking the cells to each other.

Although it is theoretically possible that our quadroma produces up to 10 different immunoglobulin molecules due to shuffling of the heavy and light chains (25), our purified product is very efficient in retargeting T-cells to B-cells (Figure 4). This was expected because in general the percentage of unwanted heavy / light chain combinations is low because each heavy chain has the highest affinity for its own light chain. Moreover, the most important and most abundant 'contaminants', the parental monospecific species, were certainly not isolated (see Fig 1). The experiments above and the high retargeting efficiency therefore suggest that the BsAb is present in abundant amounts. The high cytotoxicity observed may be due to optimal T-cell activation caused by costimulatory signals delivered by the B-cells and / or by the increased sensitivity of the B-cells for FasL after binding of anti-CD20 Abs. Although we did not address this question in this study it will be interesting to investigate if in the bispecific setting, the apoptosis inducing signal induced upon anti-CD20 crosslinking plays an important role or whether the T-cell-mediated signal is far more efficient. Another important question is if BIS20x3 is more efficient in treating lymphomas than CD19xCD3 BsAbs. Several CD19xCD3 BsAbs have been described recently (26-28) in several Ab-formats (quadroma-derived, bi-specific single-chain and diabody respectively). The findings that CD20 seems to be higher expressed on B-cells compared to CD19 and that CD20 is not internalized after Ab binding in contrast to CD19 Ab complexes are often used as arguments for the use of CD20 Abs (e.g. 29). In vivo comparison of CD3xCD19 Abs with BIS20x3 would be very informative in this matter.

The application of T-cell retargeting anti-CD20 Abs in therapies for B-cell malignancies may be an improvement upon rituximab-based therapies. Our finding that T-cells are very efficiently activated in a setting in which B-cells are present is very promising in this respect. This characteristic is probably caused by the fact that unlike most other cancer cells, malignant B-cells may be capable to function as antigen-presenting cells (24). Also the recent finding that after crosslinking of anti-CD20 Abs on B-cells Fas is up-regulated in these cells (6) could have major implications for T-cell retargeting-based lymphoma therapies, because this will make the B-cells even more sensitive for FasL on activated T-cells.

Acknowledgements

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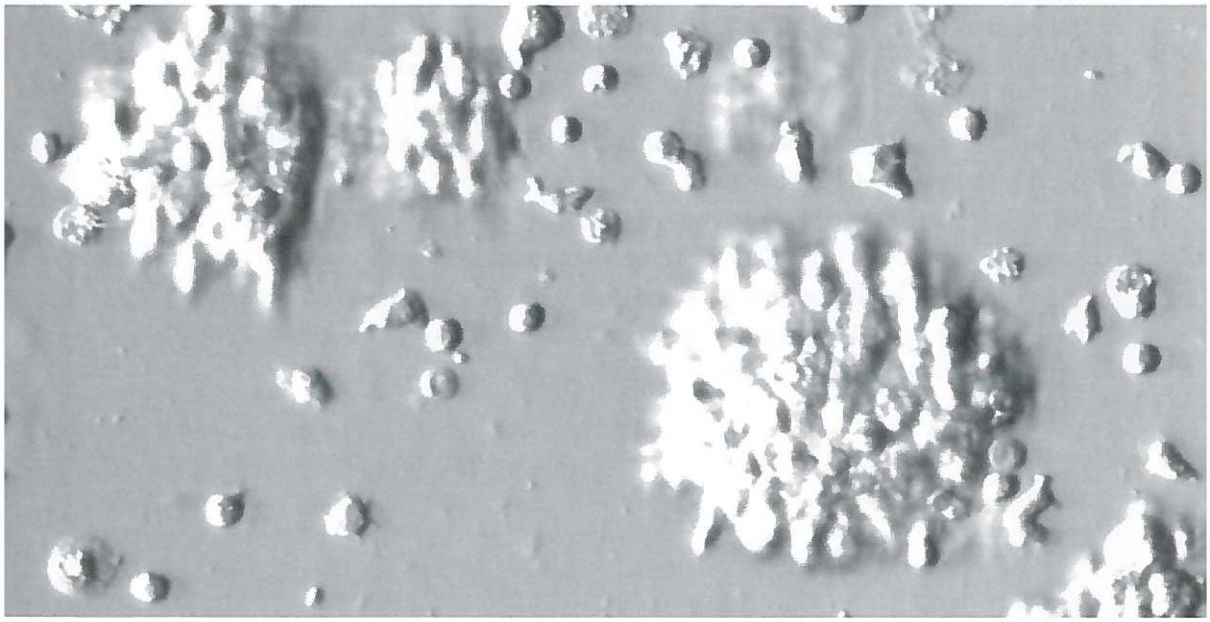
Chapter 3



The Role of B-cell Mediated T-cell Costimulation in the Efficacy of the T-cell Retargeting Bispecific Antibody BIS20x3.

The Journal of Immunology (2004), 173: 6009-6016.

Abstract

In this study, we investigated the role of the naturally occurring B-cell mediated T-cell costimulation in the anti-tumour efficacy of the bispecific antibody BIS20x3. BIS20x3 has a dual specificity for both CD20 and CD3ε and has previously been shown to effectively direct the lytic potential of cytolytic T-cells towards malignant, CD20-positive B-cells. BIS20x3 instigated T-cell / B-cell interaction caused a dose dependent activation of T-cells which was 30 times stronger when compared to T-cell activation induced by monovalent anti-CD3ε Abs. The activation of T-cells by BIS20x3 and B-cells appeared functional and resulted in the rapid induction of high lytic potential in freshly isolated peripheral T-cells. BIS20x3 mediated T-cell / B-cell interaction resulted in a significant upregulation of ICAM-1 on B-cells and the activation of T-cells was found to be dependent on the interaction of ICAM-1 with LFA-1 and trans-activation by the NF-κB pathway. Also, the lytic potential of freshly isolated T-cells activated via BIS20x3 appeared to be dependent on NF-κB signaling in the target B-cells. Interestingly, the here described costimulatory signaling effects appeared specifically related to the targeting against CD20 since targeting against CD19, by a CD3xCD19 directed BsAb, was significantly less effective in inducing T-cell activation and T-cell mediated B-cell lysis. Together these results demonstrate that the malignant B-cells actively contribute to their own demise upon CD20 directed BsAb mediated T-cell targeting.

Introduction

Treatment of non Hodgkin's Lymphoma (NHL) patients with the chimeric anti-CD20 Ab rituximab (Mabthera/Rituxan/IDEC-C2B8, IDEC Pharmaceuticals (SanDiego) and Genentech (San Fransisco), USA) results in promising response rates. In patients with relapsed follicular B-cell lymphoma, up to 50% complete and partial responses are reported (1,2). Used as a first line treatment in patients with follicular lymphoma, rituximab induced a 73% response rate (3). Despite these initial positive responses, rituximab treatment is not curative and the median progression-free survival (PFS) in responders is limited to 10-13 months (1,4,5). Rituximab induces cell death by means of antibody dependent cell-mediated cytotoxicity (ADCC), complement mediated cell lysis and via direct apoptotic signaling, the latter induced by crosslinking of rituximab on the surface of the B-cell, via Fc-receptor positive cells (6-10). The causes of relapse and the mechanism of cellular resistance to rituximab are not fully understood to date, but some groups have reported a possible decreased induction of ADCC and complement mediated cell lysis, for example via the increased expression of complement inhibiting molecules or polymorphism in IgG Fc-receptors (11-15). To improve the response rate of anti-CD20 Ab therapy we developed a bispecific Ab (BsAb) named BIS20x3. BIS20x3 combines the antigen-specificity for CD20 on B-cells with the antigen-specificity for CD3ε on T-cells, within one Ab molecule (16). In contrast to rituximab, this BsAb uses the patients T-cell compartment for its anti-tumor effect by activating and retargeting T-cells to CD20-positive B-cells, independent of the TcR/MHC-specificity of the T-cells.

Previously we demonstrated that BIS20x3 induces efficient T-cell activation and allows retargeting of T-cells to CD20-positive B-cells, resulting in efficient killing of CD20-positive B-cells. In addition to this, BIS20x3-mediated crosslinking of CD20 at the cell surface of B-cells, induced significant apoptosis in the B-cell lymphoma cell line Ramos (16). In the present study we investigated the mechanism of T-cell activation mediated by BIS20x3 and B-cells. "Full blown" T-cell activation via BIS20x3 targeted B-cells appeared to involve integrin mediated costimulation of the T-cells and required a functional NF-κB pathway in the B-cells. In addition, this B-cell mediated costimulation of T-cells was specifically related to the targeting against CD20. Together, these data demonstrated that B-cells are not just passive targets for T-cell retargeting therapy. Instead, BIS20x3 allows active participation of the CD20-positive target B-cells in the functional activation of T-cells and this ultimately resulted in the effective BsAb-mediated eradication of the malignant B-cells.

Materials and Methods

Antibodies and reagents.

BIS20x3 was produced and purified by IQ Products (Groningen, The Netherlands) as described previously (16). The immunoreactivity of both binding moieties within BIS20x3 was characterised using flowcytometric analysis, using secondary antibodies against the murine IgG1 (CD20) and IgG2b (CD3) chains within the BsAb which were purchased from IQ Products. The presence of these IgG1 and IgG2b chains and the homogeneity of the preparation was also demonstrated using gel electrophoresis of the heavy and light chains (results not shown). The anti-CD3ε Ab 37-6673 (17) was a kind gift from Dr. R.A. van Lier (Academic Medical Centre, Amsterdam, The Netherlands). The hybridoma cell-line B-ly1 producing the IgG1 anti-CD20 Ab B-ly1 (18) was generated in our department. $F(ab')_2$-fragments of GaM-IgG Abs (GaM) used for crosslinking of BIS20x3 or anti-CD3 Abs were obtained from Jackson Laboratories (West Grove, PA, USA). The anti-LFA-1 (CD11a) Ab (clone L15) was a kind gift from Prof. Dr. C. G. Figdor (Dept. Tumor Immunology, Nijmegen, The Netherlands). The anti-ICAM-1 (CD54) Ab (clone Hu5/3) was provided by Dr. M. A. Gimbrone Jr (Dept. of Pathology, Brigham and Woman's Hospital, Boston, USA). The CD3xCD19 BsAb was kindly provided by Dr. G. Moldenhauer (Dept. of Molecular Immunology, DKFZ, Heidelberg, Germany) (19). Commercial APC-, FITC- or PE-conjugated Abs (against CD3, CD20, CD25 and CD54) used for FACS analysis were obtained from IQ Products. Hygromycin B was obtained from Invitrogen (Breda, The Netherlands), Calcein-AM from Molecular Probes (Leiden, The Netherlands) and recombinant human IL-2 from EuroCetus (Amsterdam, The Netherlands). Lymphoprep was purchased from Nycomed Pharma AS (Oslo, Norway), Lympho-kwik (T) from One Lambda Inc. (Canoga Park CA, USA), serum- and phenol-free medium (X-VIVO™) from Bio-Whittaker (Verviers, Belgium) and Triton X-100 from Sigma-Aldrich BV (Zwijndrecht, The Netherlands).

Cells.

The CD20-positive Burkitt's Lymphoma cell line Ramos was obtained from the ATCC (Rockville, MD, USA). Ramos-IkBα_{ND} (Ramos cells transduced with the non-degradable inhibitor of NF-κB; IkBα_{ND}) and Ramos$_{LZRS}$ (Ramos cells transduced with an empty LZRS vector as a control) were generated by retroviral transduction as described previously (20). The CD20-positive B-cell lines Raji (Dr. Melvin Little, University of Heidelberg, Heidelberg, Germany), and Daudi (Prof. Dr. C.J.M. Melief, University Medical Center, Leiden, The Netherlands), the EBV-immortalized human B-cell line JY (Dr. L. Bakker, Dept. Tumor Immunology, Nijmegen, The Netherlands) and the T-cell leukemia cell line Jurkat AM/T (Dr. P. Schrier, University Hospital

Leiden, Leiden, The Netherlands) were cultured in RPMI 1640 culture medium (containing 25 mM Hepes and L-glutamin), supplemented with 14% heat-inactivated FCS from Bodinco BV (Alkmaar, The Netherlands), 1 mM sodium pyruvate, 2 mM L-glutamin, 0.5 mM β-mercaptoethanol and 0.1 mg/ml gentamycin sulphate obtained from Bio-Whittaker (Verviers, Belgium) and 0.02 µg/ml fungizone (Bristol-Meyers, Woerden, The Netherlands). All cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Culture medium of the Jurkat AM/T cell line was supplemented with 0.5 mg/ml hygromycin B.

Detection of T-cell activation.

T-cell activation was determined using the Jurkat T-cell subline Jurkat AM/T as described previously (16,21). Briefly, in each experiment $1.0x10^6$ Jurkat AM/T cells were activated in 1.0 ml medium at 37°C, using the stimuli described, in the presence or absence of 0.2 $x10^6$ B-cells (Ramos, Ramos-IkBα_{ND}, Ramos$_{LZRS}$, Raji, Daudi or Jy). The B-cells were characterized by flow cytometry for CD20 and CD54 expression. To block the LFA-1/ICAM-1 interaction, Jurkat AM/T cells were pre-incubated for 30 minutes at 37°C with various concentrations of the blocking anti-LFA-1 Ab, clone L15. Ramos cells were similarly pre-incubated with various concentrations of the blocking anti-ICAM-1 Ab, clone Hu 5/3. An isotype matched sham-Ab was used as a control. T-cell activation was analysed at various time points up to 24 hours by detection of luciferase activity using the Luciferase Assay System (Promega, Leiden, The Netherlands) according to the instructions of the manufacturer. Luminescence was measured on the Anthos Lucy Microplate Luminometer and Photometer (Labtech International, Ringmer, UK). Experiments were performed in triplicate and luciferase levels were corrected for levels obtained in medium control samples.

PBMC and T-cell isolation and activation.

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized whole blood by density centrifugation using Lymphoprep. T-cells were purified from the isolated PBMC using Lympho-kwik (T), according to the protocol provided by the manufacturer. For activation, T-cells were incubated for 48 hours with or without BIS20x3 in the presence of 50 Gy irradiated-Ramos cells and 100 IU/ml recombinant human IL-2. After BIS20x3-mediated activation, the T-cells were characterized by flow cytometry for CD3 and CD25 expression and used in a cytotoxicity assay to measure lytic capacity.

Cytotoxicity assays.

Cytolytic activity of the BIS20x3 retargeted T-cells was determined using the Calcein-release assay as described (22). Briefly, target cells were resuspended in X-VIVO^{TM}-medium at a concentration of 2 x 10^6 cells/ml and incubated with 8 µM Calcein-AM for 40 minutes, after which extracellular Calcein-AM was removed by washing. For the experiments, quadruplicates of 2.0x10^4 labeled target cells and T-cells at different E/T-cell ratios were added in 200 µl medium per well to round-bottom 96 wells plates in the presence of 100 IU/ml IL-2. After two hours incubation in the absence or presence of 0.5 µg/ml of the BsAbs, cells were pelleted by centrifugation and the supernatant was transferred to a new 96-wells plate. Calcein fluorescence in the supernatant was determined using a Bio-Tek FL500 fluorescence plate reader (BioTek® Instruments Inc, Burlington, USA; excitation at 485 nm, emission at 530 nm). The percentage cytotoxicity was calculated from the formula: $(F_{sample\ lysis} - F_{spontaneous\ release}) / (F_{maximal\ lysis} - F_{spontaneous\ release})$ x 100% = % cytotoxicity. Maximal lysis values were obtained by addition of 0.5% Triton X-100 to labeled target cells.

Results

Resting T-cells are activated by BIS20x3 in the presence of B-cells.

To investigate whether BIS20x3 can induce T-cell activation without the use of additional costimulation, PBMC-derived T-cells were incubated with irradiated Ramos cells in the absence or presence of BIS20x3. As shown in Fig 1A, significant upregulation of the activation marker CD25 was noted on T-cells incubated with Ramos cells in the presence of BIS20x3. No upregulation of CD25 was noted on T-cells incubated with Ramos cells in the absence of BIS20x3. Incubation of isolated T-cells with Ramos B-cells in the presence of BIS20x3 resulted in the formation of large aggregates of T-cells around the B-cells along with a typical change in morphology associated with activation of the T-cells (Fig 1B). To assess the cytolytic potential of the T-cells, fresh Calcein-AM labeled Ramos cells were added to the T-cells, which had been pre-stimulated with irradiated Ramos B-cells and BIS20x3 for 48 hours. As shown in Fig 1C, T-cell pre-stimulation in the presence of BIS20x3 induced significant cytolytic potential resulting in the induction of a 45 % lysis of target-cell death at an E/T cell ratio of 30:1.

CD3-signaling in Jurkat-AM/T cells induced by BIS20x3.

To demonstrate the capacity of BIS20x3 to activate T-cells via CD3ε-crosslinking, the NFAT-luciferase reporter T-cell line Jurkat AM/T was used, in which T-cell activation can be determined quantitatively by measuring luciferase activity. Fig 2A shows an increase in T-cell activation upon binding of increasing concentrations of BIS20x3 to CD3ε on the surface of Jurkat-AM/T cells in the presence of GaM-fragments, resulting in a 3 fold increase in luciferase activation. To attain cell-mediated CD3 crosslinking via the BsAb, CD20-positive Ramos cells were added to the Jurkat-AM/T cells in the presence of BIS20x3. Again, a dose dependent increase in T-cell activation was observed upon stimulation with increasing amounts of BIS20x3 (Fig 2B). Notably, the maximum level of T-cell activation was 25 fold higher than the activation levels obtained with the same concentration of BIS20x3 (1.0 µg/ml) crosslinked by GaM-fragments alone. Interestingly, using saturating concentrations of BIS20x3, T-cell activation decreased, indicating that the interaction between B- and T-cells, as shown in Fig 1B, contributed significantly to the efficiency of T-cell activation. Similar to Ramos, three other CD20-positive B-cell lines, Raji, Jy and Daudi also mediated T-cell activation in a BIS20x3 restricted manner (data not shown).

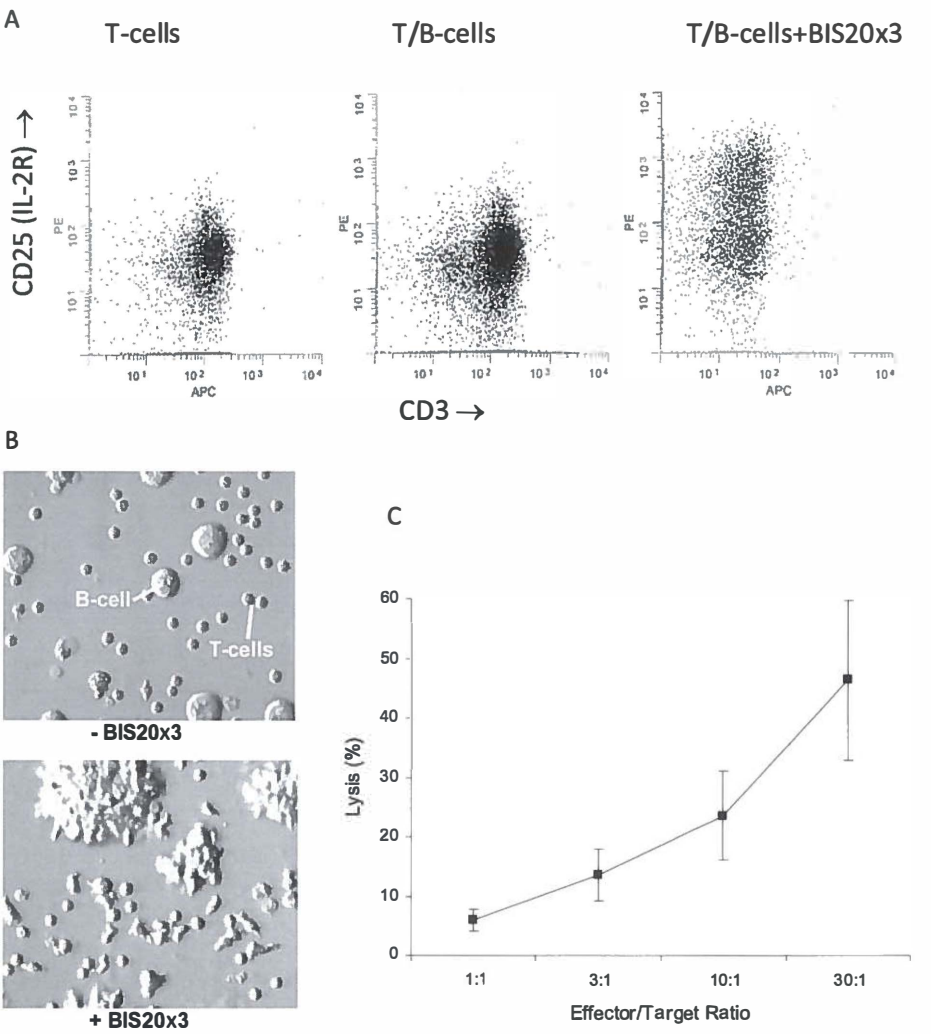


Figure 1. Freshly isolated T-cells can be activated and retargeted to CD20-positive B-cells in the presence of BIS20x3 and can induce lysis in vitro in B-cell target cells. PBMC-derived T-cells were incubated for 48 hours with irradiated Ramos cells (50 Gy) in a 1:5 B-cell/T-cell ratio with or without BIS20x3 (0.5 μg/ml). (A) Cells were harvested and stained with anti-CD3 and anti-CD25 APC- and PE-conjugated Abs. CD25 expression is significantly increased on T-cells when cultured with Ramos cells in the presence of BIS20x3. (B) Pictures were taken from samples containing Ramos and T-cells alone or Ramos and T-cells incubated with BIS20x3. Aggregate formation and typical changes in cell morphology associated with T-cell activation are induced. (C) After the above described pre-incubation, fresh Calcein-AM (8 μM) labeled Ramos cells were added to the T-cells in different ratios and incubated for two hours. Lysis of Ramos cells was determined by measuring Calcein fluorescence in the supernatant. The percentage lysis obtained in Ramos cells cultured with BIS20x3 was corrected for the percentage lysis found in matching samples without BIS20x3. The mean result of quadruplicate assays is displayed for each ratio along with the SEM.

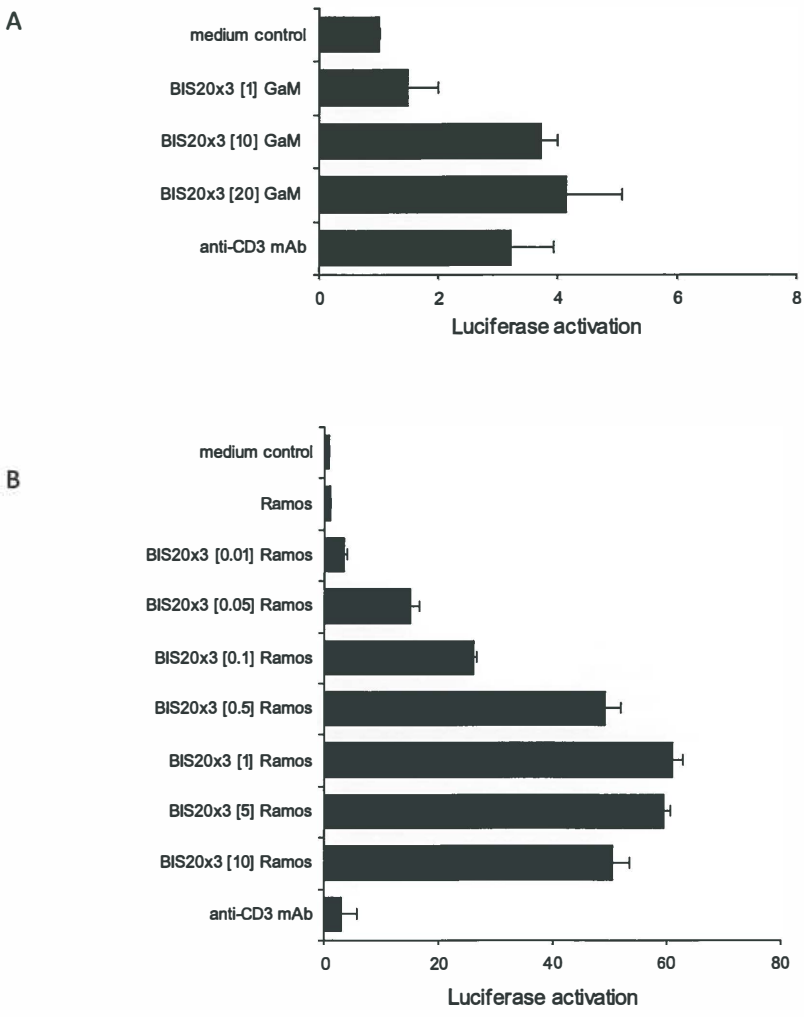
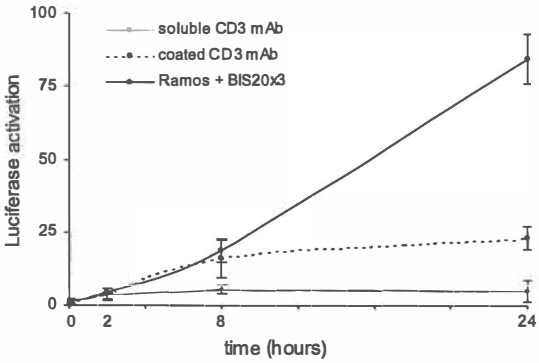


Figure 2. BIS20x3 is capable of activating T-cells via CD3-crosslinking. (A) Jurkat-AM/T cells, containing the NFAT-luciferase reporter gene, were cultured with different concentrations BIS20x3 and 7.5 µg/ml crosslinking GaM for 20 hours. Luciferase activity of the medium control was set at 1.0 arbitrary unit and each condition was corrected for (divided by) the medium control accordingly. The mean percentage of triplicate assays (+/- SD) is displayed for each condition. As a positive control, T-cells were stimulated with 5 µg/ml Ab 37-6673 (anti-CD3ε). (B) Ramos cells and BIS20x3 were added to the Jurkat-AM/T cells in a B/T-cell ratio of 1:5 to induce cell-mediated crosslinking of CD3ε instead of direct anti-CD3ε Ab mediated crosslinking via GaM. Monospecific anti-CD3ε Abs (5 µg/ml) were used as a positive control. As a negative control Ramos and Jurkat-AM/T cells were co-cultured without BIS20x3. The mean percentage of triplicate assays is displayed for each condition along with the SD. Note that the X-axis in (B) is 10-fold higher than in (A).

A



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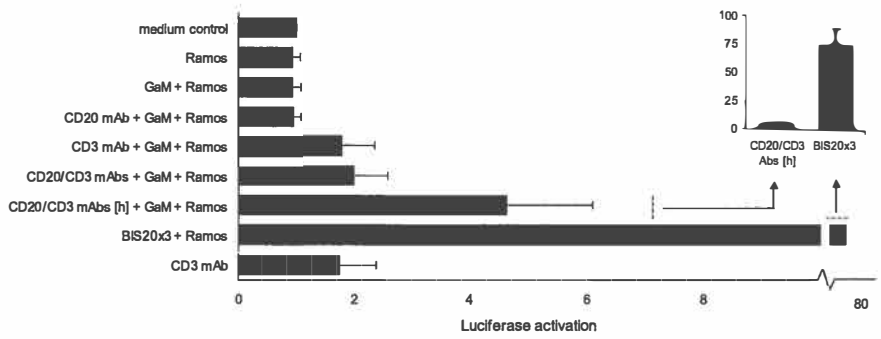

Figure 3. T-cell activation by BIS20x3 in the presence of B-cells is more effective than differential forms of CD3ε-crosslinking alone. (A) Jurkat-AM/T cells (1.0 x 10^6) were cultured for 0, 2, 8 and 24 hours in the presence of soluble anti-CD3ε Ab (5 μg/ml), anti-CD3ε Abs pre-coated to the bottom of the plate for 24 hrs in PBS (5 μg/ml) or in the presence of Ramos cells (0.2x10^6) and BIS20x3 (0.5 μg/ml). T-cell activation, determined by luciferase activity, was corrected for the medium control. The mean level of activation is displayed for each condition and time point along with the SD. B, Jurkat-AM/T cells were cultured for 20 hrs with 0.5 μg/ml B-ly1 (parental anti-CD20 Ab) or 0.5 μg/ml 37-6673 (parental anti-CD3ε Ab) or a combination of these Abs in the presence of 7.5 μg/ml crosslinking GaM-fragments. In one sample the concentrations anti-CD20 Abs and anti-CD3 Abs were increased to 10 μg/ml using 25 μg/ml GaM-fragments as indicated [h]. In all samples except the medium control and the positive CD3ε Ab control (5 μg/ml), Ramos cells were added to the Jurkat AM/T cells in a 1:5 ratio. Luciferase activity of the medium control was adjusted at 1.0 arbitrary unit and results were corrected for the medium control accordingly. The mean result of triplicate assays is displayed for each condition along with the SD. Note that the bar for BIS20x3 mediated T-cell activation is interrupted. A direct comparison between BIS20x3 activated T-cells in the presence of B-cells and T-cell activation as obtained by activation with the high concentration [h] anti-CD20/CD3 Abs plus GaM is displayed in the inserted graph.

The effects of differential CD3ε-crosslinking on the levels of T-cell activation.

To further investigate the cause of the significant activation of T-cells, different forms of CD3ε ligation were compared. Soluble monospecific anti-CD3ε Abs minimally increased the level of activation in Jurkat-AM/T cells during the first eight hours, whereas for Jurkat-AM/T cells in plates pre-coated with anti-CD3ε Abs T-cell activation kept increasing for at least 24 hours (Fig 3A). However, coated anti-CD3ε Abs caused T-cell activation at a level far below the level that could be reached via stimulation through BIS20x3 and Ramos cells. To demonstrate that the stimulatory effect of BIS20x3 on T-cells was not just the additive result of simultaneous CD3ε and CD20 signaling, Jurkat AM/T cells were incubated with both B-ly1 (the parental anti-CD20 Ab) and 37-6673 (the parental anti-CD3ε Ab), in the presence of Ramos cells (Fig 3B). Crosslinking the anti-CD20 Ab B-ly1 on Ramos cells was not sufficient to induce T-cell activation. In concordance with the results described in Fig 2A and 3A, the crosslinked monoclonal anti-CD3ε Ab could only induce minor levels of T-cell activation. To mimic the dual binding properties of BIS20x3, both parental Abs and crosslinking GaM-fragments were added simultaneously to the Ramos/Jurkat-AM/T mixture, thus bridging both cell lines via GaM-fragments. This led to an increased activation of the T-cells, especially when high (10 μg/ml) concentrations of the Abs were used. However, as shown in Fig 3A, BIS20x3 induced a 15-fold more effective T-cell activation. These results demonstrate that the efficient activation by crosslinking was not merely caused by CD3ε crosslinking on T-cells in the presence of B-cells. The close cell-cell interaction between the B- and T-cells as induced by BIS20x3 binding seems a prerequisite for this effect.

LFA-1/ICAM I interaction is involved in T-cell costimulation induced by BIS20x3 and Ramos cells.

We hypothesized that the mechanism of efficient T-cell activation via BIS20x3 takes place through the natural interaction of costimulatory molecules such as LFA-1 and ICAM-1 present on T- and B-cells. It has been described that costimulatory molecules in B-cells are upregulated upon activation of the B-cell (23,24). We first determined if BIS20x3-mediated activation of B-cells influenced expression levels of ICAM-1 on the surface of these cells. Fig 4A shows that Ramos cells display a constitutive expression of ICAM-1 (CD54), which could be significantly upregulated through activation with BIS20x3 and Jurkat-AM/T-cells. Similar increases in ICAM-1 expression were observed in Raji, Daudi and JY cells (not shown). The induction of costimulatory molecules on B-cells suggested an active role for the BIS20x3 targeted B-cells in the activation of T-cells. Next, we investigated the functional significance of the involvement of ICAM-1. Fig 4B shows that inhibition of the LFA-1/ICAM-1 interaction by blocking Abs reduced T-cell activation to 50% using an

anti-LFA-1 Abs and to 40% for the anti-ICAM-1 blocking Abs. An isotype matched control Ab did not decrease T-cell activation.

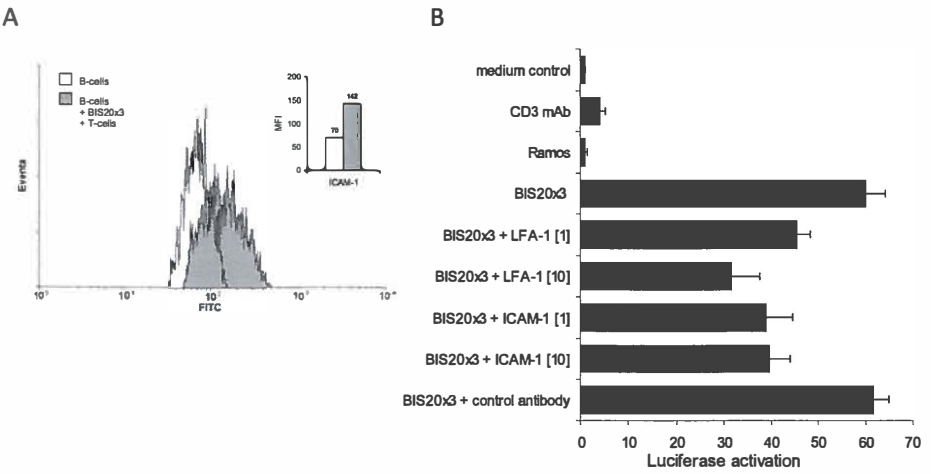

Figure 4. T-cell activation by BIS20x3 in the presence of B-cells involves a functional interaction between LFA-1 and ICAM-I. (A) Jurkat-AM/T cells and Ramos cells were cultured in a 5:1 ratio for 20 hours at 37°C with or without 0.5 μg/ml BIS20x3 present. Cells were harvested and Ramos cells were stained with FITC- and PE-conjugated anti-CD20 and anti-CD54 Abs. Ramos cells cultured with BIS20x3 and T-cells displayed an increased expression of CD54. In the insert, CD54 expression is expressed in MFI (mean fluorescence intensity). The figures show one of two representative experiments. (B) Jurkat-AM/T cells were pre-incubated for 30 minutes with different concentrations (1.0 and 10 μg/ml) blocking anti-LFA-1 (clone L15) Abs or Ramos cells were pre-incubated for 30 minutes with the same concentrations anti-ICAM-1 (clone Hu5/3) Abs. As a control both cell types were also pre-incubated with 10 μg/ml isotype-specific control Abs. After pre-incubation, Ramos cells and BIS20x3 (0.5 μg/ml) were added to the Jurkat-AM/T cells in a 1:5 B-cell / T-cell ratio in all samples except the medium control and the positive CD3ε mAb control (5 μg/ml) and samples were cultured for 20 hours in a total volume of 1.0 ml. Luciferase activity was determined and corrected for the medium control. The mean result of triplicate assays along with the SD is displayed for each condition.

B-cell mediated costimulation of T-cells involves NF-κB gene transcription and is CD20 dependent.

It is known that the regulation of many immunostimulatory signals in lymphocytes, for example ICAM-1 expression on B-cells, is under control of NF-κB-signaling (25-28). We therefore investigated the involvement of the NF-κB-pathway in the activation of B-cells to become efficient T-cell costimulatory cells. For this purpose we made use of an NF-κB defective Ramos subline (Ramos-IkBα$_{ND}$), in which overexpression of a non-degradable form of IkBα prevents nuclear translocation and trans-activation of NF-κB (20). We hypothesized that B-cells deficient in NF-κB translocation are less potent in costimulating Jurkat AM/T cells. ICAM-1 expression

on the Ramos-IkBα_{ND} cells was not increased upon incubation with BIS20x3 and T-cells (data not shown). Inhibition of NF-κB translocation in the B-cell resulted in a less potent activation of Jurkat-AM/T cells (Fig 5A). When using Ramos cells transduced with the empty vector LZRS (Ramos$_{LZRS}$), T-cell activation levels at 24 hours were comparable to those shown for the parental Ramos cells. These findings show that NF-κB regulated gene-transcription in the B-cell is important for efficient costimulation of T-cells in the presence of BIS20x3, eventually leading to the activation of cytotoxic T-cells. We also compared the effects of BIS20x3 with another, well-defined B-cell (CD19) targeting, BsAb named CD3xCD19. Identical amounts of CD3xCD19 or BIS20x3 were added to Jurkat-AM/T cells in the presence of Ramos cells. Although the same concentration of Abs and the same amounts of B- and T-cells were used, CD3xCD19 Abs induced less T cell activation as can be seen in Fig 5B. Finally, we measured the lytic capacity of freshly isolated T-cells pre-incubated with either Ramos-IkBα_{ND} cells or the parental Ramos B-cells in the presence of BIS20x3 or CD3xCD19. T-cells incubated with Ramos-IkBα_{ND} cells and BIS20x3 or CD3xCD19 displayed less aggregate formation. The lytic capacity of T-cells stimulated by BIS20x3 and Ramos-IkBα_{ND} was decreased when compared to pre-incubation with parental Ramos cells and BsAb (Fig 5C). The lytic capacity of freshly isolated T-cells was also strongly decreased when CD3xCD19 BsAbs were used instead of BIS20x3. T-cells pre-incubated with CD3xCD19 induced only minimal lysis of both Ramos and Ramos-IkBα_{ND} cells, which indicates that B-cells triggered with CD3xCD19 Abs were not as efficiently activated to costimulate T-cells as were B-cells triggered with BIS20x3. These findings reinforce the hypothesis that CD20-mediated B-cell activation through the NF-κB pathway is essential for the induction of costimalory signals and leads to an efficient increase of the activation level of T-cells.

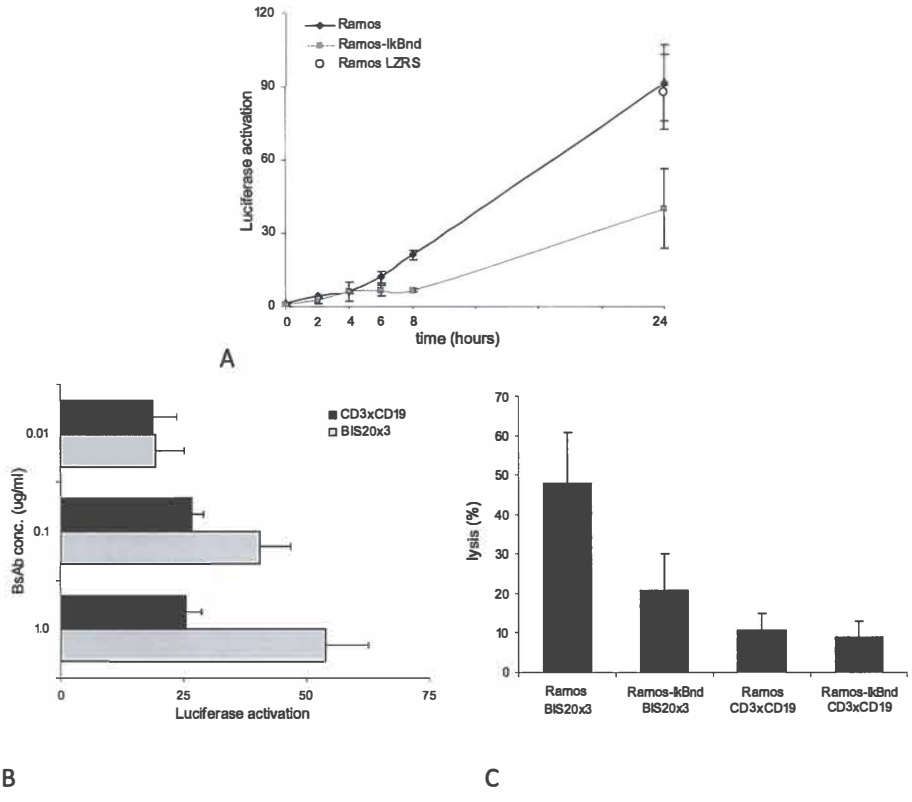

Figure 5. T-cell activation by B-cells in the presence of BIS20x3 involves NF-κB and is CD20 dependent. (A) Jurkat-AM/T cells were cultured in the presence of Ramos or Ramos-IkBα_{ND} cells in a 5:1 ratio and BIS20x3 (0.5 μg/ml) in a total volume of 1.0 ml. As an additional control, Ramos$_{LZRS}$ (empty vector) cells were cultured with the T-cells and BIS20x3 for 24 hours. Luciferase activity was measured and corrected for medium control samples. The mean level of luciferase activation is displayed for each time point and condition along with the SD. (B) Jurkat-AM/T cells were incubated for 20 hours with Ramos cells in a 5:1 ratio in the presence of the BsAbs BIS20x3 or CD3xCD19 in concentrations increasing from 0.01 to 1.0 μg/ml. Luciferase activity was measured and corrected for medium control samples. The mean levels of luciferase activation along with the SD are displayed. (C) T-cells isolated from PBLs were pre-stimulated as described with 0.5 μg/ml BIS20x3 or CD3xCD19 Abs and irradiated Ramos or Ramos-IkBα_{ND} as costimulatory cells. After pre-incubation, new Calcein-AM labeled Ramos and Ramos-IkBα_{ND} cells were added to the T-cells in a 30:1 effector/target ratio and incubated with fresh BsAbs (0.5 μg/ml) for two hours. Lysis of Ramos or Ramos-IkBα_{ND} cells was determined by measuring Calcein fluorescence in the supernatant. The percentage lysis obtained in cells cultured with either BIS20x3 or CD3xCD19 is corrected for the percentage lysis found in matching samples without BsAb. The mean result of three independent assays is displayed along with the SEM.

Discussion

The chimeric Ab rituximab is the most effective anti-cancer Ab described to date. Still, rituximab therapy is not curative and virtually all patients relapse. It is observed that relapsed lymphomas express higher levels of complement inhibitory proteins or deficient Ab dependent tumour-cell lysis. These findings suggest that therapeutic outcome may improve if the need for the complement system can be circumvented and/or the lytic hit delivered to the target cells can be enforced. To meet these objectives we designed BIS20x3, which is not dependent on components of the humoral immune response but derives its anti-tumour effect from the cytocidal activity of retargeted T-cells in a CD20-restricted manner resulting in an efficient eradication of CD20-positive B-lymphocytes.

A number of BsAb formats have been described over the last decade and in vivo anti-tumour activity has been shown in some cases in experimental clinical treatment settings (29-31). Many studies have described the necessity of costimulation as a prerequisite for effective BsAb mediated T-cell retargeting, posing a limitation with respect to the clinical effectiveness of many of the BsAb described to date (32,33). One significant advantage of BIS20x3 lies in the fact that BIS20x3 targets B-cells, which are considered professional APCs and are thus intrinsically capable of relaying costimulatory signals to T-cells. We have demonstrated that cell-cell interactions between Ramos B-cells and T-cells, mediated through binding of BIS20x3, leads to a highly efficient activation of the T-cells (Fig 1-3). This is not a finding unique for Ramos cells, but was noted in several B-cell lines, suggesting that these B-cell lymphoma cell lines, and presumably B-cell lymphomas in general, have the capacity of triggering T-cells with costimulatory signals.

We had two alternative hypotheses for the efficient T-cell activation by BIS20x3. First, the possibility existed that the length of the CD3ε-stimulus determined the activation state of the T-cells. It has been shown that specific binding of an Ab to CD3ε leads to internalization of the TcR/CD3-Ab complex and eventually to the extinction of the T-cell activation signal (34). Simultaneous binding of BIS20x3 to B-cells could probably prevent TcR/CD3 internalization, thus leading to longer stimulation and higher levels of T-cell activation. Indeed, coating of monovalent anti-CD3ε Abs resulted in increased activation of T-cells when compared to soluble anti-CD3ε Abs. However, this activation did not reach the efficiency by which BIS20x3 was shown to activate T-cells in the presence of B-cells. This suggested that, although the mechanism of CD3ε binding and crosslinking is important for

CD3ε-mediated T-cell activation, in the case of BIS20x3 other mechanisms of T-cell triggering play a major role.

The second hypothesis was that, upon binding of BIS20x3, B- and T-cells converge and costimulatory molecules such as the integrins LFA-1 and ICAM-1 may interact, leading to reciprocal signaling within both the B- and T-cells. The interaction of these costimulatory molecules may be allowed to take place in a physiological manner because the membrane distance, necessary for these interactions, matches the predicted distance between cells bridged by BIS20x3. Normally, the T-cell antigen receptor interacts with MHC-bound antigen, which leads to an intercellular membrane distance of ~ 7 nm (35-37). IgG molecules (as for example BIS20x3) are known to have a spatial distance between their Ab binding sites of 5.5-7 nm. (38) Therefore, the bridge between the T- and B-cells formed by BIS20x3 spans the same distance as in a physiological T-cell/APC interaction.

The initial purpose of BIS20x3 is to attract T-cells to malignant B-cells and simultaneously induce T-cell activation via CD3 triggering, thus circumventing TcR-restriction and mimicking the activation of the natural immune synapse. CD20 is described to interact with MHC class II on various B-cell lines. (39,40). This suggests that CD20 might be part of the larger SMAC (supra molecular activation cluster) complex, which is critically involved in MHC/TcR signaling and T-cell activation. We hypothesized that BIS20x3 binding to CD20 is expected to induce costimulatory molecules to interact with their counterparts in the vicinity of the TcR, thereby efficiently enhancing the activation of the T-cell.

We have shown that ICAM-1 expression is increased upon CD20 stimulation and we demonstrated that the interaction of ICAM-1 and LFA-1 contributes significantly to the efficient activation of T-cells (see Fig 4). An important implication of this finding is that the malignant target B-cells play an active role in the activation of the T-cells resulting in the concomitant eradication of the B-cells themselves.

As it is known that activation of B-cells (via the BcR, CD40 or MHC class II) or the expression of costimulatory molecules can be regulated by NF-κB-signaling (25,28,41,42), we investigated whether the BIS20x3 mediated costimulatory signals were also under control of the NF-κB pathway. Pathways that regulate NF-κB mediated gene-transcription have been described to be involved in CD20 signaling (43). For example, the Src-family protein-tyrosine kinase (PTK)-signaling pathway is described to be shared in several B-cell activation routes including CD20 and NF-κB signaling (40,44-47). We demonstrated that the BIS20x3 mediated costimulatory effects observed in the activated Ramos B-cells are under the control of NF-κB

trans-activation. In addition, we showed that Jurkat-AM/T cells were less efficiently activated using Ramos-IκBα$_{ND}$ cells (Fig 5A). Also the lytic capacity of freshly isolated T-cells was significantly decreased upon pre-incubation with BIS20x3 and Ramos-IκBα$_{ND}$ cells instead of "normal" Ramos cells. These results underline the importance of signaling through the NF-κB pathway in the target B-cells when BIS20x3 is applied (Fig 5C).

To investigate if the BIS20x3 incited costimulatory capacity of B-cells was restricted to CD20-targeting, another B-cell directed BsAb, CD3xCD19, was used. Both BsAbs are directed against the CD3ε chain of the TcR-CD3 complex and stimulate T-cells with similar characteristics and kinetics. As such the CD3ε binding parts of the two BsAb were considered comparable in physiological terms. We demonstrated that the CD3xCD19 BsAb induced significantly less T-cell activation and, as a result, did not yield the cytolytic potential in resting T-cells as obtained with BIS20x3 (Fig 5B and C). This suggested that CD20 targeting BsAbs are more efficient in eliciting costimulatory signals on B-cells. These findings were not merely the result of different expression rates of CD19 and CD20 molecules on the surface of the Ramos cells, because we found that the level of Jurkat-AM/T T-cell activation was not associated with the number of CD20 molecules on various B-cell lines (results not shown). This indicated that variation in the level of T-cell activation is not a direct quantitative effect of the amount of targeted CD20 molecules on the surface of B-cells. Also the affinity of antigen binding by the BsAbs does not seem to be intervening with our findings, considering that saturating concentrations BsAbs are used (1.0 µg/ml or higher) resulting in significant differences between CD20 and CD19 targeting. However it can not be excluded that under non-saturating conditions, differences in affinity between both BsAbs might be a co-factor in cellular costimulatory effects. Furthermore, in contrast to CD20, binding of antibodies to CD19 leads to the internalization of CD19, which suggests that in a clinical setting such BsAbs may not be available for T-cell targeting. CD19-targeting Abs might therefore be considered as a preferred reagent for delivery of cytotoxic agents rather than for T-cell retargeting therapy (48-50). Moreover, it was described that in vivo anti-tumor effects of unconjugated anti-CD20 mAbs are superior to those of anti-CD19 mAbs (51). Because BIS20x3 targets to CD20 instead of CD19, it is likely that this BsAb could perform well in clinical settings without the need for additionally applied costimulatory reagents such as e.g. CD28 Abs. In vivo comparison of CD3xCD19 Abs with BIS20x3 should be performed to prove the hypothesized higher clinical efficacy of BIS20x3 over CD3xCD19.

Upon transformation and progression of malignancies unique tumour antigens may develop and anti-tumour specific blocking Abs could interfere with therapeutic Ab

binding or be involved in tumour proliferation. However, to our knowledge this phenomenon has not (yet) been demonstrated to occur in the case of CD20 directed therapies such as with rituximab which may relate to the fact that the CD20 receptor neither is a unique tumour antigen nor is it known to be associated with transformation or tumour progression. No studies exist that show either interference of anti-CD20 binding or functional hindrance of anti-CD20 directed treatment modalities by CD20 directed blocking host Abs. We therefore do not expect this phenomenon to be a major problem for the clinical applicability of a CD20 targeting BsAb like BIS20x3.

In conclusion, the results described in this paper show that BIS20x3 is a very potent reagent for the activation and retargeting of T-cells to CD20-positive malignant B-cells. We demonstrate for the first time that targeting against CD20 via BIS20x3 induces a process of NF-κB dependent costimulation, which allows efficient and functional T-cell activation. The role of B-cells as professional antigen presenting cells is thus efficiently utilized by BIS20x3, which makes this Ab a particularly promising new candidate for the treatment of B-cell malignancies and warrants further investigation of this treatment modality in experimental clinical treatment settings.

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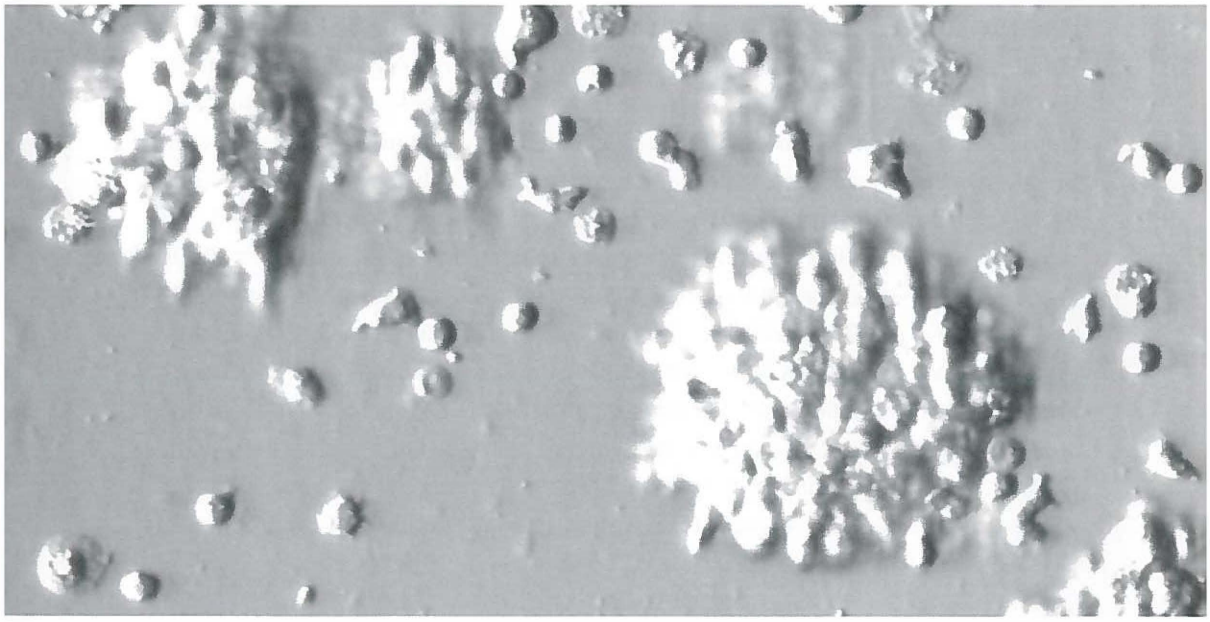
Chapter 4



Fas receptor clustering and involvement of the death receptor pathway in Rituximab-mediated apoptosis with concomitant sensitization of lymphoma B-cells to Fas-induced apoptosis.

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Abstract

Antibody binding to CD20 has been shown to induce apoptosis in B-cells. In the present study we demonstrate that rituximab sensitizes lymphoma B-cells to Fas-induced apoptosis in a caspase-8 dependent manner. To elucidate the mechanism by which rituximab affects Fas-mediated cell death, we investigated rituximab-induced signaling and apoptosis pathways. Rituximab-induced apoptosis involved the death receptor pathway and proceeded in a caspase-8 dependent manner. Ectopic overexpression of FLIP (the physiological inhibitor of the death receptor pathway) or application of zIETD-fmk (specific inhibitor of caspase-8, the initiator-caspase of the death receptor pathway) both specifically reduced rituximab-induced apoptosis in Ramos B-cells. Blocking the death receptor ligands FasL or TRAIL, using neutralizing Abs, did not inhibit apoptosis, implying that a direct death receptor/ligand interaction is not involved in CD20-mediated cell death. Instead, we hypothesized that rituximab-induced apoptosis involves membrane clustering of Fas molecules that leads to formation of the death inducing signaling complex (DISC) and downstream activation of the death receptor pathway. Indeed, Fas coimmune precipitation experiments showed that, upon CD20-crosslinking, FADD and caspase-8 were recruited into the DISC. Additionally, rituximab induced CD20 and Fas translocation to raft-like domains on the cell surface. Further analysis revealed that, upon stimulation with rituximab, Fas, caspase-8 and FADD were found in sucrose-gradient raft fractions together with CD20. In conclusion, in this study, we present evidence for the involvement of the death receptor pathway in rituximab-induced apoptosis of Ramos B-cells with concomitant sensitization of these cells to Fas-mediated apoptosis via Fas multimerization and recruitment of caspase-8 and FADD to the DISC.

Introduction

The efficient B-cell depletion induced by the chimeric anti-CD20 Ab rituximab *in vivo* is due to simultaneous activation of multiple cell death-inducing mechanisms. It is well established that rituximab can activate both Ab-dependent cellular cytotoxicity and complement-dependent cytotoxicity (1,2). In addition, crosslinking of rituximab, for example via FcR-positive cells, leads to the activation of an intracellular signaling cascade which results in B-cell death by apoptosis (3,4). Rituximab has been described to sensitize lymphoma cells to standard chemotherapeutic reagents used for treatment of Non-Hodgkin's lymphoma and, very recently, to Fas receptor-induced apoptosis (5-7). These observations are of significant clinical importance as exemplified by the implementation of combination therapies with rituximab, which have shown to prolong progression free and overall survival (8,9). Despite its undisputed clinical relevance, the exact way by which rituximab exerts its apoptosis inducing activities is still not understood.

Several signaling molecules and pathways have been described to be involved in rituximab-induced apoptosis. Crosslinking of anti-CD20 Abs on B-cells leads to translocation of CD20 to lipid microdomains know as rafts, followed by activation of members of the src-family of tyrosine kinases, elevation in intracellular Ca^{2+}, and phospholipase Cγ activation (10,11). In addition, CD20 crosslinking results in a rapid upregulation of Bax, changes in RNA level of c-*myc* and Berg36, activation of MAPK family members p44 and 42 and increased AP-1 DNA binding activity (12). p38 MAPK and STAT3 protein activity have been reported to be inhibited as a result of CD20 crosslinking by rituximab, subsequently downregulating the anti-apoptotic proteins Bcl-xL, Bcl-2, and inducing Apaf-1 (13-15). Other anti-apoptotic signaling pathways, including the ERK 1/2 and the NFκB pathway have been reported to be inhibited by rituximab, resulting in the sensitization of various B-cell lines to chemotherapy (5,16) and Fas receptor (7)-mediated apoptosis induction. *In vivo*, in chronic lymphocyte leukemia (CLL) patients, rituximab treatment results in activation of caspase-9 and -3, followed by the execution of apoptosis (4). Although it is generally believed that the mitochondrial pathway, activated via caspase-9, is the main apoptotic cascade induced by rituximab, other routes, activated via caspase-7 and -8, have been reported as well (17,18).

To further delineate the significance of apoptosis inducing and apoptosis sensitizing signaling characteristics of rituximab, we studied the possible involvement of the death receptor pathway in rituximab-mediated apoptosis induction. We used the Burkitt lymphoma cell line Ramos, and retrovirally transduced derivatives of this

cell line, expressing high levels of FLIP and XIAP as a model. This allowed us to differentiate between the involvement of the DISC and caspase-9 and -3 mediated apoptosis. Both the death receptor and the mitochondrial apoptosis pathway were triggered upon crosslinking of rituximab, resulting in the activation of caspase-9 as well as caspase-8. Rituximab-induced activation of caspase-8 was preceded by a death receptor /ligand-independent clustering of Fas in membrane microdomains and by formation of the DISC. The lateral membrane reorganisation of Fas and the formation of a functional DISC by rituximab were instrumental to a significant part of rituximab-induced apoptosis and resulted in concomitant sensitization to apoptosis induction via the Fas receptor. In conclusion, in this study we demonstrate a novel, initiating role for the death receptor pathway in the process of rituximab-mediated cell death, which provides a new, membrane dependent, mechanism to overcome Fas resistance and sensitize B-cells to the death receptor mediated apoptosis.

Materials and Methods

Cells and culture media.

The Burkitt lymphoma type, germinal center B-cell line Ramos-RA1 (ATCC) and retrovirally transduced derivatives of this cell line were cultured in RPMI 1640 culture medium (containing 25 mM Hepes and L-glutamin) obtained from BioWhittaker (Verviers, Belgium) supplemented with 10% heat-inactivated FCS from Bodinco BV (Alkmaar, The Netherlands), 1 mM sodium pyruvate, 2 mM L-glutamin, 0.5 mM β-mercaptoethanol and 0.1 mg/ml gentamycin sulphate all obtained from BioWhittaker and 0.02 µg/ml fungizone (Bristol-Meyers, Woerden, The Netherlands). All cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Antibodies and reagents.

Rituximab was kindly provided by Roche Nederland BV (Woerden, The Netherlands). F(ab')2-fragments of goat-anti-human-IgG (further denoted as crosslinker or CL) used for crosslinking of rituximab, and the anti-BcR Ab (goat-anti-human IgM) were obtained from Jackson Laboratories (West Grove, PA, USA). Rabbit-anti-human DNA fragmentation factor (DFF) and rabbit-anti-human active caspase-3 Abs were obtained from Pharmingen (BD Biosciences, Alphen aan den Rijn, The Netherlands). Anti-actin Abs were purchased from ICN Biochemicals (Zoetermeer, The Netherlands) and hybridoma supernatant containing mouse-anti-human-caspase-8 Ab, (clone C15, (19)) was kindly provided by Dr. H. Walczak (DKFZ, Heidelberg, Germany). Mouse-anti-human-caspase-9 and rabbit-anti-human Bid Abs were obtained from Cell Signaling Technology, Inc (Beverly, MA, USA). Mouse-anti-human-FADD Abs were purchased from Transduction Laboratories (BD Biosciences, Alphen aan den Rijn, The Netherlands). Horseradish peroxidase (HRP)-conjugated secondary Abs for Western blotting were obtained from DAKO (Glostrup, Denmark) or from Santa Cruz Biotechnology, Inc. (Tebu-Bio, Heerhugowaard, The Netherlands). Rabbit-anti-human poly(ADP-ribose) polymerase (PARP) Abs, rabbit-anti-human Lyn Abs and the blocking anti-human-Fas Ligand (FasL) Ab NOK-1 were also obtained from Santa Cruz. Soluble human recombinant Super*Killer*TRAIL, the TRAIL neutralizing Ab 2E5, the mouse-anti-human-CD95/Fas Ab APO1-3, the mouse-anti-human-FLIP Ab NF6 and soluble human recombinant FasL APO-1L were purchased from Alexis (Kordia BV. Leiden, The Netherlands). As a crosslinking enhancer of FasL APO-1L, the mouse-anti-FLAG M2 Ab was purchased from Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands). The mouse IgM anti-human Fas antibody 7C11 was purchased from Immunotech (Beckman Coulter, Mijdrecht, The Netherlands). The caspase-inhibitors zVAD-FMK and zIETD-FMK were obtained from Calbiochem (Omnilabo

International, Breda, The Netherlands). Triton X-100, propidium iodide (PI), RNAse-A, Puromycin, PMSF, sodium deoxycholate, trichloroacatic acid and Tween-20 were obtained from Sigma-Aldrich Chemie BV. Poly-acrylamide and Bradford reagent were purchased from Bio-Rad (Hercules, CA, USA). Skim milk was obtained from Difco (BD Biosciences, Alphen aan den Rijn, The Netherlands). The restriction enzymes *XhoI* and *NotI* were purchased from Gibco-BRL (Invitrogen, Breda, The Netherlands). Protein-A Sepharose 4 Fast Flow beads were purchased from Pharmacia Biotech (AB, Uppsala, Sweden), and complete protease inhibitors cocktail from Roche Diagnostics.

Generation of retroviral particles encoding the short form of cellular FLIP (cFLIP$_S$).
The human cFLIP$_S$ (Genbank accession no. U97075) gene was obtained using mRNA isolated from the testicular germ cell tumor cell line Tera, kindly provided by Dr. S. de Jong (University Medical Center Groningen, The Netherlands). RNA was isolated using the StrataPrep® Total RNA microprep kit (Stratagene, La Jolla, CA, USA). The Sensiscript™ Reverse Transcriptase kit (Qiagen, Westburg BV, Leusden, Belgium) was used to generate cDNA. The forward primer used for amplification of cFLIP$_S$ was 5'-ATCTCGAGATGTCTGCTGAAGTCATCCAT-3'. The sequence of the backward primer was 5'-ATGCGGCCGCTCACATGGAACAATTTCCAAG-3'. In these primers *XhoI* and *NotI* restriction sites were introduced to enable shuttling into the retroviral vectors. PCR cycling was performed from 95°C to 55°C to 68°C and back, using eLONGase® (Gibco-BRL). PCR products were isolated from agarose gels using the QIAquick cel extraction kit (Qiagen), subcloned into the TOPO® XL PCR-cloning vector (Invitrogen) and sequence-analyzed. Subsequently, the genes were shuttled into the retroviral vector LZRS-pBMN-IRES-eGFP, kindly provided by Dr. H. Spits (NKI, Amsterdam, The Netherlands), which was derived from LZRS-lacZ(A) (20). Amphotropic Phoenix packaging cells were transfected using Fugene (Roche Diagnostics) as described by the manufacturer. Transfected packaging cells were selected by culturing in the presence of 1 µg/ml puromycin. Retroviral particle batches were obtained by isolating culture supernatant from large scale cultures (without puromycin) and stored at -80°C.

Transduction of Ramos cells.
Ramos cells were transduced by co-culturing $2x10^6$ Ramos cells (in 2 ml) with 3 ml retroviral supernatant. The cultures were expanded and transduced cells were sorted on Green Fluorescent Protein (GFP)-fluorescence by flow cytometry using the MoFlo (Modular Flow Cytometer, Cytomation Inc., Fort Collins, CO, USA). The XIAP overexpressing Ramos cell line was generated according to a similar protocol, as described previously (21). Overexpression of cFLIP$_S$ and XIAP was confirmed by Western blot (results not shown).

Induction of apoptosis by crosslinking rituximab on Ramos B-cells.

One day prior to each experiment, Ramos cells or cells from its retrovirally transduced derivatives were harvested and diluted to a concentration of $0.25x10^6$ cells/ml. The following day, cells were harvested and incubated for 30 minutes with 10 µg/ml rituximab at a concentration of $5.0x10^6$ cells/ml in culture medium at 37°C. Hereafter, the cells were washed to remove unbound rituximab and plated at a concentration of $1.0x10^6$ cells/ml in 24-well plates. Crosslinking was achieved by adding CL in a final concentration of 15 µg/ml. To inhibit caspase activity 20 µM (final concentration) zVAD-FMK or zIETD-FMK was added in the experiments.

Detection of DNA fragmentation.

DNA fragmentation was analysed as described previously (22,23). In short, cells were washed in PBS containing 1% bovine serum albumine (BSA) and resuspended in a hypotonic buffer (0.1% Na-citrate, 0.1% Triton X-100, 0.1 mg/ml RNAse-A) containing PI at a final concentration of 50 µg/ml. PI-fluorescence was detected at 625 nm by flow cytometry (Epics Elite, Coulter-Electronics, Hialeah, FL, USA).

Preparation of cell lysates for Western blotting.

Cells were lysed in lysis buffer (30 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 10% glycerol, 1mM PMSF, supplemented with complete protease inhibitors cocktail) for 15 minutes at 4°C. After sonification on ice for 5 seconds (Bandelin sonopuls, Berlin, Germany), cell debris was pelleted by centrifugation at 10 000xg for 10 minutes at 4°C. The supernatant was collected and protein concentrations of each sample were determined using Bradford reagent (24). Samples for SDS-PAGE and Western blotting were prepared by mixing with an equal volume of 2x sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-β-mercaptoethanol, 0.002% bromophenol blue) and heating the samples for 5 minutes at 95°C. SDS-PAGE size-separated proteins were transferred onto Bio-Rad Trans-blot nitrocellulose membrane for immunodetection according the manufacturer's protocol.

Protein detection.

Blots were blocked for at least 1 hour at room temperature in blocking buffer (PBS, 5% skim milk, 0.1 % Tween-20). Primary Abs were diluted in blocking buffer as recommended by the supplier and blots were incubated for at least 1 hour. Subsequently, the blots were washed in PBS containing 0.1% Tween-20 and secondary antibody (HRP-conjugated, 1:2000 diluted in blocking buffer) was added for 30 minutes. Finally, after washing, the conjugates were visualized using Lumi-Light^{PLUS} Western blot substrate (Roche Diagnostics) and Kodak Biomax MR-1 film (Sigma-Aldrich Chemie BV).

Immune precipitation of CD95/Fas.

CD20 crosslinking was induced as described above, using 10 µg/ml rituximab for the 30 minutes pre-treatment period and 15 µg/ml CL for crosslinking on $10x10^6$ Ramos cells per sample at a concentration of $1.0x10^6$ cells/ml. Cells were harvested at various time points, washed using cold PBS and gently lysed in immunoprecipitation buffer (30 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1mM PMSF, supplemented with complete protease inhibitors cocktail). Protein concentration was determined and aliquots of total lysates were used separately for Western blot analysis. The remaining samples were all adjusted to equal concentrations in 1 ml precipitation buffer and Fas was immune precipitated for 1 hour using 2 µg/ml anti-CD95/Fas Ab (APO 1-3). Precipitated Fas, together with co-precipitated proteins of the DISC, was extracted from the samples using protein A Sepharose 4 Fast Flow beads. The beads plus the precipitate were mixed with an equal volume of 2x sample buffer, heated for 5 minutes at 95°C and spun down at 70xg for 3 minutes at 4°C. Finally, the supernatant, including the precipitated proteins, was carefully separated from the beads, run on a SDS-poly-acrylamide gel and transferred onto a Bio-Rad Trans-blot nitrocellulose membrane for immunodetection.

Analysis by fluorescence microscopy.

To perform fluorescent staining of CD20, rituximab was labeled with tetramethylrhodamine (TRITC) according to the manufacturer's protocol (Molecular Probes, Invitrogen, Breda, The Netherlands). Ramos cells ($1.0x10^6$ cells/ml) were cultured for 15 minutes in presence or absence of TRITC-labeled rituximab (10 µg/ml), washed using cold PBS and fixated with 3.5% paraformaldehyde (Merck BV, Darmstadt, Germany) during 10 minutes on ice. Samples were subsequently incubated on ice with 2 µg/ml anti-Fas Abs (7C11) and 5% Goat-anti-Mouse Alexa Fluor 488-conjugated Abs (Molecular Probes), each for 20 minutes after which two rounds of washing with cold PBS were performed. Untreated cells were directly fixed with paraformaldehyde and subsequently stained with both anti-Fas and rituximab-TRITC. After staining cells were analysed using a Quantimet 600S digital analysis system (Leica Microsystems Nederland BV, Rijswijk, The Netherlands).

Isolation of lipid rafts by sucrose-gradient ultracentrifugation.

Ramos cells $5.0x10^6$ cells/ml were incubated with 10 µg/ml rituximab for 15 minutes in culture medium at room temperature. After incubation, medium was removed by centrifugation and the cells were dissolved in ice-cold Tris-buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA) with 1% Triton X-100 and 1 µM aprotinin, leupeptin, and pepstatin A, all obtained from Sigma-Aldrich Chemie BV.

The solution was vortexed and kept on ice for 30 minutes. The lysate was homogenized by passing it twenty times through a 21-gauge needle. To isolate lipid rafts, lysates were mixed with an equal volume of ice cold 80% sucrose in Tris-buffer and overlaid with a 35% sucrose and a 5% sucrose layer in Tris-buffer. The gradients were centrifuged in a Beckman SW41 swing-out rotor at 40 000 rpm for 18 hours at 4°C, to allow equilibration.

After centrifugation, 10 fractions were taken from top to bottom of the gradients. They were transferred to micro test tubes and vortexed. Protein content of the fractions was determined and 10 µg of protein was taken from each fraction and adjusted to 10 µg/ml. Five microliters of sodium deoxycholate 25 mg/ml was added to each tube, followed by 5 minutes incubation on ice. Subsequently, 60 µl of trichloroacetic acid 100% (w/v) was added and the samples were vortexed, incubated on ice for 10 minutes and centrifuged for 15 minutes, 4500xg at 4°C. Supernatant was removed and the precipitated proteins in the pellets were dissolved in 20 µl sample buffer, neutralized for 10 minutes with ammonia vapor and stored at -20°C. Western blotting and protein detection were performed as described.

Results

Rituximab sensitizes Ramos cells to Fas mediated apoptosis.

Rituximab sensitizes B-cells to other apoptosis inducing drugs like cyclophosphamide, doxorubicin, vincristine, prednisone, paclitaxel and cisplatin (9,18,25). In addition, it was recently described that rituximab is able to revert Fas resistance (7,26). We treated Ramos cells with combinations of rituximab, goat-anti-human-IgG (CL), and anti-Fas Abs. As positive controls for the mitochondrial and the death receptor pathway, Ramos cells were treated with anti-BcR Abs (IgM) or soluble TRAIL. Upon crosslinking, up to 55% apoptosis was induced by rituximab. Without crosslinking, no apoptosis was induced by rituximab. Thus, although the death receptor pathway is functional, it appeared that Ramos cells are relatively resistant to Fas-induced apoptosis. Interestingly, in the absence of crosslinking secondary Abs, rituximab significantly increased apoptosis induction via Fas. Upon treatment with rituximab (without secondary crosslinking), Fas resistance was abrogated and up to 45% apoptosis was induced by anti-Fas Abs (Fig 1A).

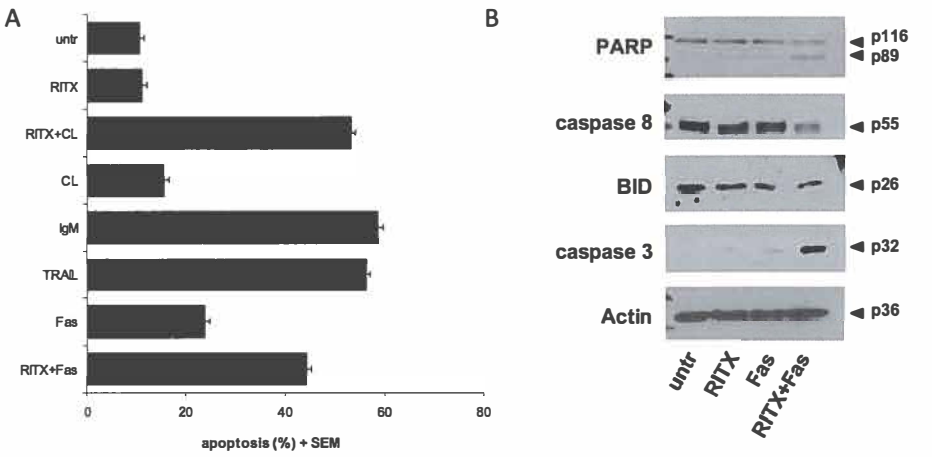

Figure 1. Rituximab sensitizes Ramos B-cells to Fas-mediated apoptosis. (A) Ramos cells ($1.0x10^6$) were pre-incubated with 10 µg/ml rituximab (RITX) for 30 minutes and subsequently incubated overnight (16 hours) with either medium control, 15 µg/ml secondary crosslinking Ab (CL) or the anti-Fas antibody 7C11 (Fas). CL alone and Fas alone were added as controls. To have an independent positive control for both mitochondrial and death receptor mediated apoptosis, Ramos cells were incubated with 5 µg/ml anti-BcR-Abs (IgM) and 500 ng/ml soluble Super*Killer*TRAIL (TRAIL). DNA fragmentation was determined by means of PI-staining to measure the percentage of apoptotic cells. Experiments were performed in quadruplicate and the mean percentage apoptosis is displayed along with the SEM. (B) Ramos cells ($1.0x10^6$) were cultured for 16 hours with rituximab, Fas or the combination as described before. Western blot lysates were prepared and blotted and blots were stained for PARP cleavage (118 kDa and its 89 kDa cleavage product), pro-caspase-8 (55 kDa), Bid (26 kDa), pro-caspase-3 (32 kDa) and the housekeeping protein Actin (36 kDa).

In the presence of additionally applied secondary rituximab crosslinking Abs, apoptosis further increased to 62%, which is approximately similar to the level of apoptosis found after treatment with crosslinked rituximab without additionally applied anti-Fas Abs. Western blot analysis revealed that cell death via sensitization to Fas involved PARP cleavage, activation of caspase-8 by cleaving its 55 kDa proform, activation of caspase-3 and also cleavage of Bid (Fig 1B).

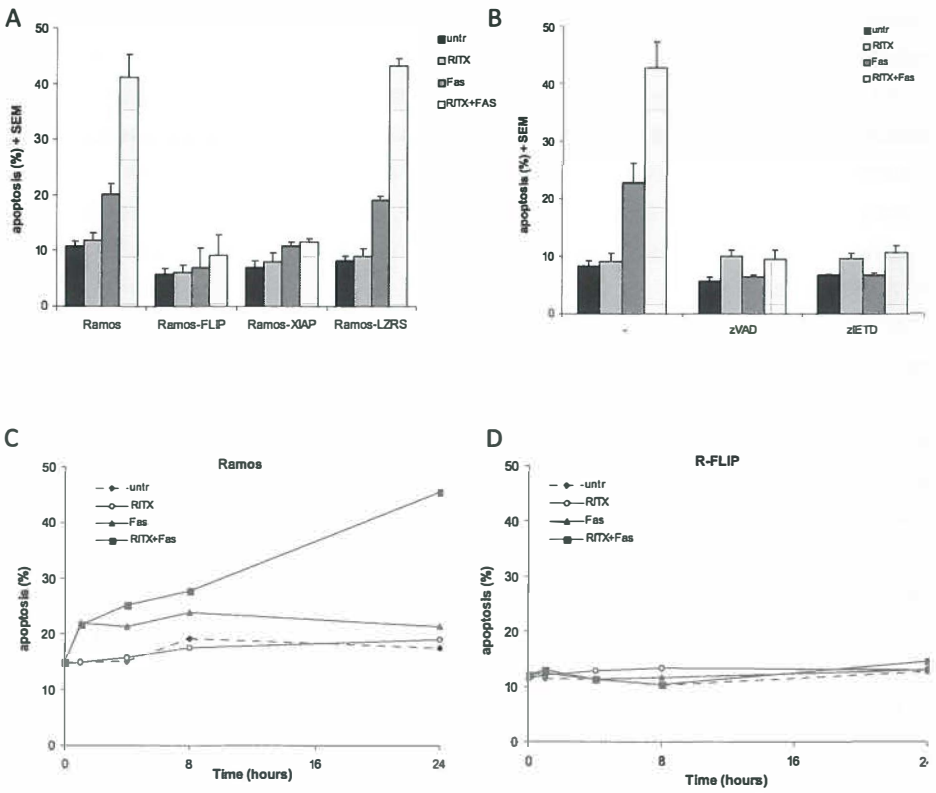

Figure 2. Rituximab-mediated Fas sensitization is caspase-8 dependent. (A) Ramos cells, the Ramos subline cells Ramos-XIAP, Ramos-FLIP and the negative (empty vector) control derivative Ramos-LZRS (all $1.0x10^6$) were incubated overnight with 10 µg/ml rituximab, 2 µg/ml Fas or the combination of both. DNA fragmentation was determined to measure the percentage of apoptotic cells. Experiments were performed in quadruplicate and the mean percentage apoptosis is displayed along with the SEM. (B) Ramos cells ($1.0x10^6$) were incubated as described before with rituximab, Fas or the combination of both. Caspase activation was inhibited with 20 µM of the general caspase inhibitor zVAD-FMK or the caspase-8 specific inhibitor zIETD-FMK. The percentage apoptotic cells was determined after 16 hours measuring DNA-fragmentation and the mean percentage is displayed together with the SEM. (C and D) Ramos cells and the Ramos-FLIP cells were cultured with rituximab, Fas or the combination for different time periods. After each period of incubation, apoptosis levels were defined in the samples by determining DNA fragmentation. One example of three separate experiments is displayed in both graphs.

Fas sensitization by rituximab is dependent on caspase-8 activation.

Rituximab is described to activate signaling cascades including the mitochondrial apoptosis pathway, whereas Fas triggering classically induces the death receptor pathway. To explore the mechanism of rituximab-induced sensitization to Fas-mediated apoptosis, two Ramos-derived cell lines, overexpressing ectopic FLIP or XIAP (Ramos-FLIP and Ramos-XIAP), were used. FLIP overexpression specifically blocks the activation of the death receptor apoptosis pathway at the level of caspase-8 activation, while XIAP prevents apoptosis downstream from mitochondrial damage and death receptor apoptosis at the level of caspase-9 and -3. All cell lines were incubated over night with rituximab, anti-Fas Abs and the combination thereof. As shown in Fig 2A, Fas resistance could be overcome by rituximab-mediated sensitization in both the parental Ramos cell line and the empty vector control, Ramos-LZRS. However, apoptosis at the level of DNA fragmentation was almost completely blocked in both Ramos-FLIP and Ramos-XIAP. In accordance, inhibition of caspase activation, by a pan-caspase inhibitor (zVAD-FMK) and by caspase-8 inhibition (zIETD-FMK), resulted in a complete inhibition of apoptosis induced by the combined treatment with rituximab and anti-Fas Abs, indicating that rituximab-mediated Fas sensitization is dependent on the activation of caspase-8 and its downstream effectors (Fig 2B). In time, apoptosis induced by anti-Fas Abs alone or in combination with rituximab increased to the same extend and with similar kinetics during the first one to two hours. Thereafter, Fas triggering sustained a maximum level of 20% of apoptosis, whereas the combination of rituximab and anti-Fas Abs induced a further increase in apoptosis levels up to 46%. In the Ramos-FLIP derivate, both the early and the late effects were inhibited regardless whether apoptosis was induced by anti-Fas Abs alone or in combination with rituximab (Fig 2C and D).

Rituximab induces caspase-mediated apoptosis.

Although rituximab is not known to activate the death receptor pathway, rituximab-mediated Fas sensitization appeared to depend completely on the activation of caspase-8. In addition, sensitization to Fas-induced apoptosis was observed in the absence of rituximab crosslinking, suggesting direct modulation of the Fas/death receptor complex by rituximab. To further investigate the role of the death receptor pathway in CD20-mediated apoptosis, we extensively studied the activation of caspases and the initiation of apoptosis by rituximab. Rituximab was crosslinked at the surface of Ramos B-cells and initiator caspase cleavage was monitored. Anti-IgM and soluble TRAIL were used as positive controls for the mitochondrial and the death receptor pathway respectively. Rituximab-mediated apoptosis was first detected between eight and twelve hours of incubation and increased up to 40 % after 24 hours (Fig 3A). Subsequently, Western blot analysis

was used to determine the kinetics of processing of caspase-8 and caspase-9 along with the proteolytic cleavage of DFF and PARP (Fig 3B). The kinetics of DFF and PARP cleavage were similar to the kinetics found for DNA fragmentation. A concurrent processing of both caspase-8 and caspase-9 preceded DFF and PARP cleavage as well as DNA fragmentation. Degradation of both proforms of caspase-8 and -9 could be detected as early as two hours after starting rituximab crosslinking.

The death receptor pathway plays an initiating role in rituximab mediated apoptosis

The involvement of the death receptor pathway in rituximab-mediated apoptosis induction was studied in more detail using the Ramos-FLIP and Ramos-XIAP cell lines. As shown in Fig 4A, overexpression of XIAP and, interestingly, also of FLIP, inhibited apoptosis induction by crosslinked rituximab. This strongly suggested a direct role for the death receptor pathway in rituximab-mediated apoptosis. In accordance with these results, addition of zIETD-FMK inhibited rituximab-mediated apoptosis at the level of DNA-fragmentation (Fig 4B) and PARP-cleavage (Fig 4C). zVAD-FMK (pan-caspase inhibitor) was applied as a control to confirm the involvement of caspases in general. The results presented in Fig 4A-C demonstrated that rituximab-mediated apoptosis induction is caspase-dependent and showed that the death receptor pathway is at least partially responsible for CD20-mediated cell death. It was shown by Shan *et al* (3) that apoptosis induction by fratricide, via interaction of membrane-bound FasL or soluble FasL with the Fas receptor, is not involved in CD20-mediated cell death. To confirm this finding in our experimental setting and to investigate if the interaction between another well described ligand, TRAIL, and its death receptors, plays a role in rituximab-induced apoptosis, FasL and TRAIL neutralizing Abs were added as described previously (27,28). Both Abs were capable of completely neutralizing apoptosis induced by soluble FasL and TRAIL. However, neither blocking of FasL nor blocking of TRAIL decreased the percentage of apoptotic cells induced by crosslinking of rituximab (Fig 4D), implying that rituximab-induced activation of the death receptor pathway is independent of death receptor/ligand interaction.

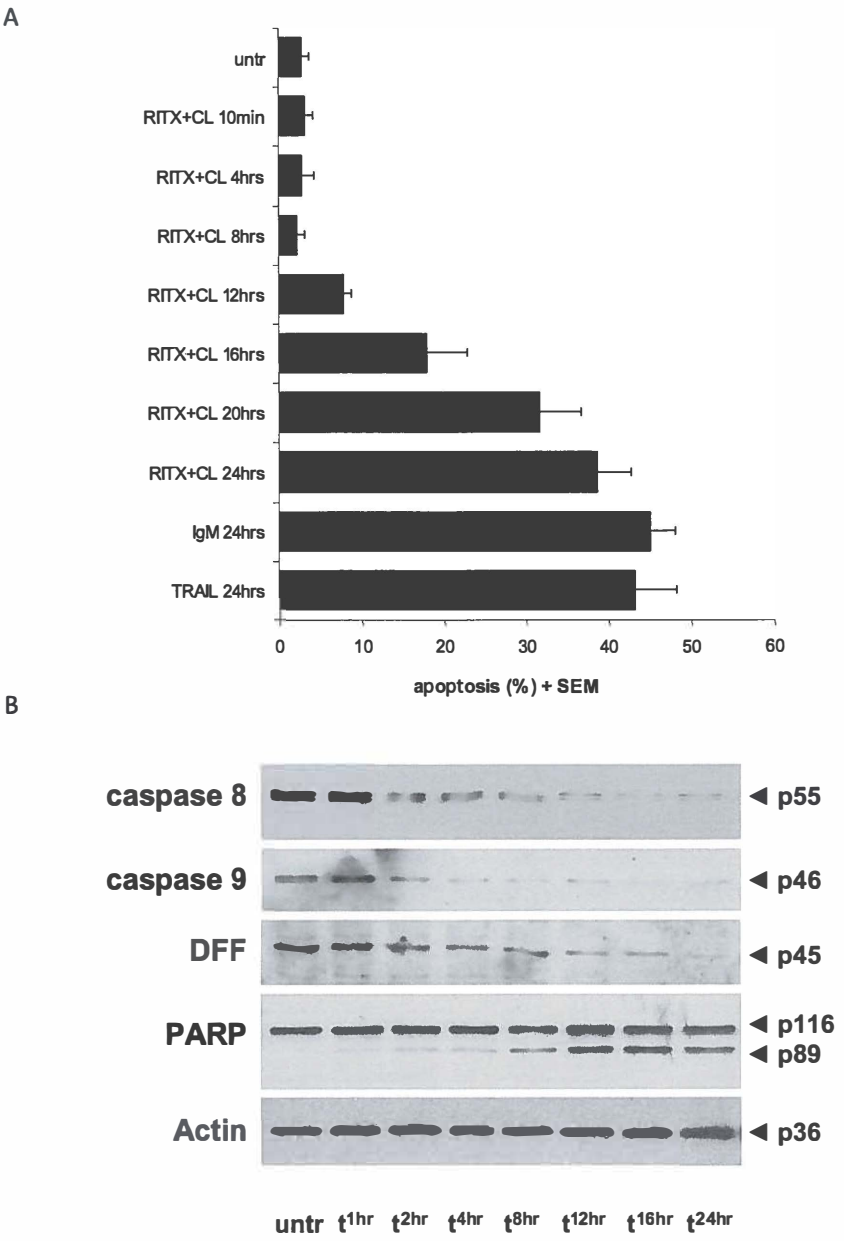

Figure 3. Rituximab-mediated apoptosis induction in time. (A) Ramos cells ($1.0x10^6$) were incubated with 10 μg/ml rituximab for 30 minutes and subsequently incubated for the described time periods with 15 μg/ml CL (crosslinking Ab). Apoptosis was quantified by determination of DNA fragmentation by PI-staining. The mean result of quadruplicate assays along with the SEM is displayed for each condition. (B) Western-blots were prepared from matching sample lysates and stained for the proforms of caspase-8 (55 kDa) and -9 (46 kDa), DFF (45 kDa) and PARP (118 kDa and 89 kDa). Actin (36 kDa) was included as a housekeeping protein to have a control for protein concentrations.

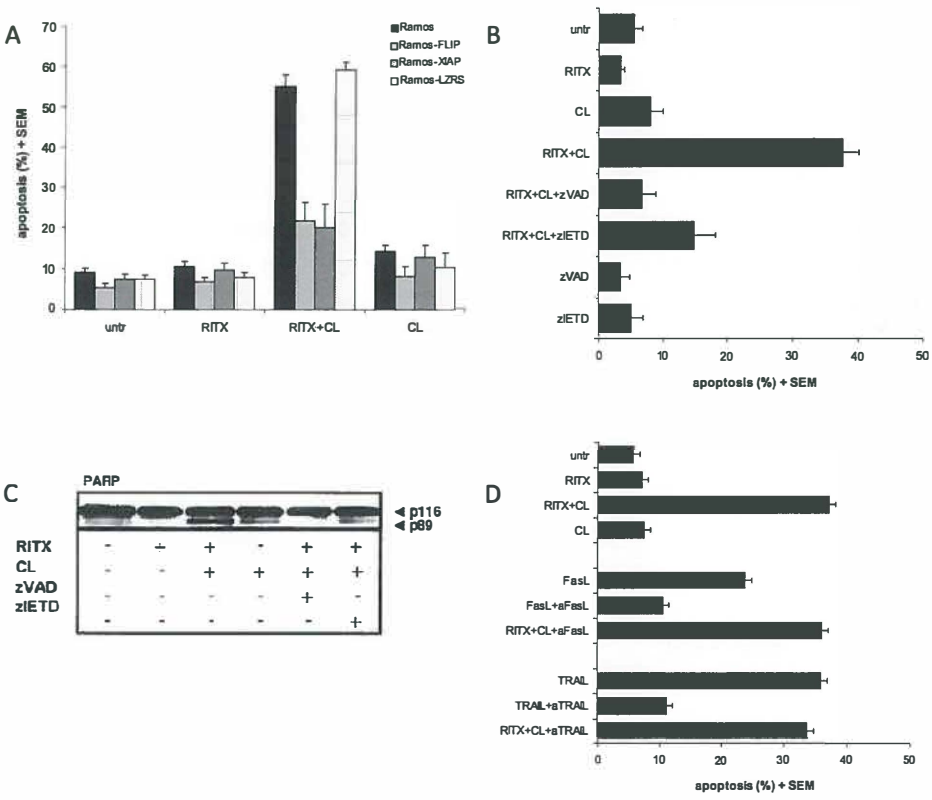

Figure 4. Rituximab-induced apoptosis is partly induced via caspase-8 activation. (A) Ramos cells, the Ramos subline cells Ramos-XIAP Ramos-FLIP and Ramos-LZRS (all $1.0x10^6$) were pre-incubated with 10 µg/ml rituximab and incubated over night with or without 15 µg/ml CL (crosslinking Ab) or with CL alone. DNA fragmentation was determined to measure the percentage of apoptotic cells. Experiments were performed in quadruplicate and the mean percentage apoptosis is displayed along with the SEM. (B) Ramos cells ($1.0x10^6$) were cultured over night with rituximab, CL or the combination. General caspase activation or specific caspase-8 activation was inhibited with 20 µM zVAD-FMK or zIETD-FMK. The percentage apoptotic cells was determined after 16 hours measuring DNA fragmentation and the mean percentage is displayed including the SEM. (C) Western-blot lysates were prepared and blotted and blots were stained for PARP cleavage (118 kDa and 89 kDa) to confirm DNA fragmentation results. (D) Ramos cells were incubated as described before with 10 µg/ml rituximab, 500 ng/ml FasL plus 2.5 µg/ml enhancer (FasL) or 500 ng/ml soluble TRAIL (TRAIL). Where indicated, Ramos cells were pre-incubated with saturating amounts of blocking NOK-1 anti-FasL (aFasL) and 2E5 anti-TRAIL (aTRAIL) Abs (10 µg/ml). DNA fragmentation was determined by PI staining and the mean result of triplicate assays along with the SEM is displayed for each condition.

Rituximab initiates formation of the DISC.

Spontaneous multimerization of CD95/Fas occurs in germinal center B-cells and has been shown to play a role in clonal selection of maturating B-cells (29). Algeciras-

Schimnich *et al* showed that multimerization of Fas is followed by the formation of micro-aggregates and receptor clustering before the actual induction of apoptosis can proceed (30). Fas-induced apoptosis is considered to be dependent on death receptor translocation to rafts where receptor clustering and DISC formation takes place (31,32). We hypothesized that rituximab-induced CD20 translocation to rafts induces concurrent clustering of multimerized Fas and subsequently allows formation of the DISC, resulting in the activation of caspase-8. We investigated whether or not the DISC was formed in Ramos cells upon CD20-triggering by immune precipitating Fas and analysis of co-precipitated proteins like caspase-8 and FADD.

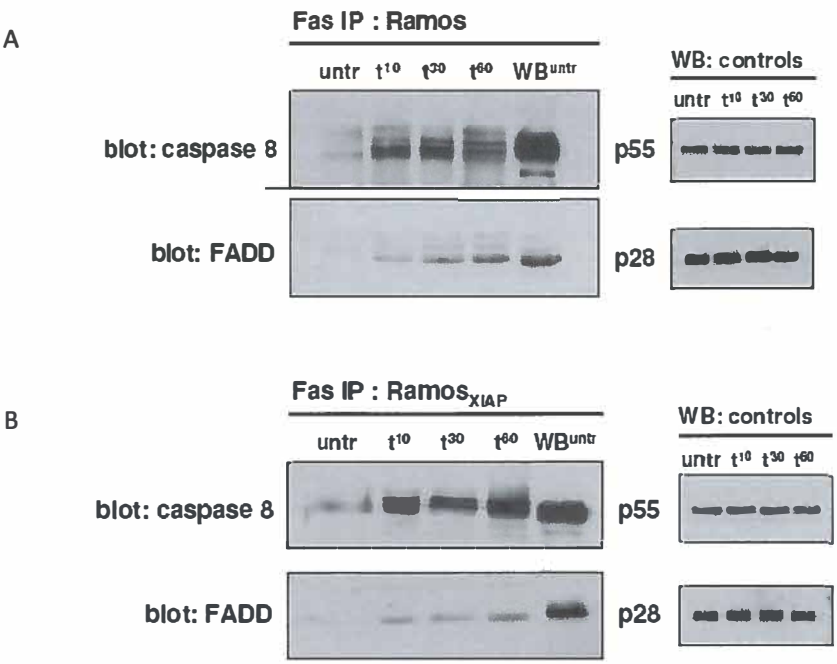

Figure 5. DISC isolation via Fas immune precipitation (IP). (A) After culturing, either in control medium (untr), or with rituximab crosslinking for 10 minutes up to 60 minutes, Ramos cells ($10.0x10^6$) were gently lysed and Fas was immune precipitated using 2.0 µg/ml anti-Fas APO1-3 Abs together with possible attached proteins of the DISC as described in *Materials and Methods*. Western-blots were prepared, followed by immunodetection of co-precipitated caspase-8 and FADD. As a positive control for the detected bands, one lane of 10 µg total lysate from untreated Ramos cells was displayed together with the IP-samples. Total lysate controls of all samples are displayed in the small pictures to confirm that differences in intensity of the detected bands are not an effect of differences in general sample protein concentration. (B) To exclude the interaction of caspase-9 and -3 activation, experiments were repeated using Ramos-XIAP cells. Both the displayed immune precipitation and the total lysate blots are representatives of three separate experiments.

As shown in Fig 5A, a time dependent recruitment of both caspase-8 and FADD to the DISC was observed in rituximab-treated Ramos cells. As a positive control for immunodetection of caspase-8 and FADD, 10 µg total lysate of untreated Ramos cells was applied on the same blot. To ensure that equal amounts of protein were used as starting material, equal fractions of the total lysates of all samples are depicted separately.Although we demonstrated that the classical DISC, initiating the death receptor pathway, was formed upon rituximab crosslinking, we wanted to make certain that CD20-triggering was the initiative stimulus for this complex to be formed. It is known that continuous processing of caspase-8 is necessary for efficient multimerization of the DISC (30). This process is likely to be catalysed by activated caspase-3, which can cleave initiator caspases like caspase-8 and is even reported to be a possible component of the DISC (33,34).
To exclude the possibility that the observed formation of the DISC via CD20 triggering is a secondary response resulting from activation of caspase-3 and/or -9, we used the Ramos-XIAP cell line in which the cleaving potential of these caspases is completely blocked (35,36). Indeed, DNA fragmentation in Ramos-XIAP cells was significantly inhibited confirming the involvement of effector caspases in the end-stage execution of rituximab-induced apoptosis (Fig 4A). However, DISC formation by recruitment of caspase-8 and FADD in Ramos-XIAP was found to be equivalent to the parental Ramos cells, suggesting that CD20 triggering is followed by an immediate formation of the DISC, which is not initiated via a downstream secondary caspase activating loop (fig. 5B).

Rituximab induces translocation of Fas to rafts.

Fas-mediated apoptosis is dependent on translocation of Fas to rafts, where receptor clustering and DISC formation takes place. We hypothesized that the initiation of DISC formation and activation of the death receptor pathway as well as Fas sensitization might result from rituximab-induced receptor translocation into rafts. We therefore examined the membrane localization of CD20 and Fas following rituximab treatment. In untreated cells, both CD20 and Fas were present on the membrane in a homogeneous, dispersed, pattern. In contrast, upon treatment with rituximab alone, this pattern changed both for CD20 and Fas into a patched pattern, indicating rapid translocation and clustering of both CD20 and multimerized Fas to raft-like domains upon CD20-stimulation (Fig 6A). To study the translocation of DISC components, like Fas, caspase-8 and FADD into rafts upon rituximab triggering, we incubated Ramos cells with rituximab and subsequently isolated lipid rafts by sucrose-gradient ultracentrifugation. Translocation of Fas, caspase-8 and FADD to rafts was determined by Western blot analysis. As a control for proper lipid raft isolation, the blots were also probed for the lipid raft marker

src-kinase Lyn (Fig 6B). Indeed, Fas, caspase-8 and FADD, along with CD20 itself, were found in the raft fractions upon treatment of Ramos cells with rituximab.

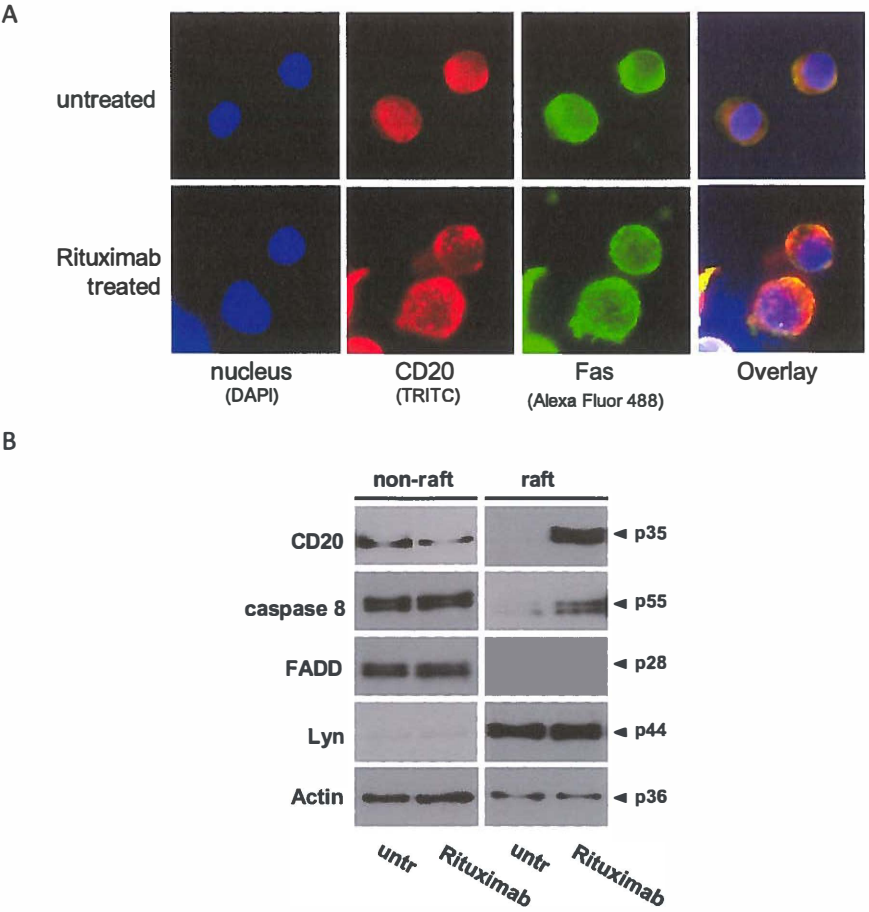

Figure 6. Rituximab induces Fas translocation into raft-like lipid domains. (A) Ramos cells ($1.0x10^6$ cells/ml) were cultured for 15 minutes in presence or absence of TRITC-labeled rituximab (10µg/ml). After fixation with paraformaldehyde, cells were stained with anti-Fas Abs (7C11) followed by goat-anti-mouse IgM Alexa Fluor 488-conjugated Abs. Untreated cells were directly fixed with paraformaldehyde and subsequently stained with both anti-Fas/anti-mouse IgM Alexa Fluor 488 and rituximab-TRITC. After staining cytospots were prepared and analyzed on a Quantimet 600S digital analysis system (Leica Microsystems Nederland BV, Rijswijk, The Netherlands). (B) Ramos cells ($5.0x10^6$ cells/ml) were incubated with 10 µg/ml rituximab for 15 minutes at room temperature. After incubation, lysates were prepared and lipid rafts were isolated by sucrose-gradient ultracentrifugation as described in Materials and Methods. After centrifugation, fractions were taken from top to bottom of the gradients and pooled into raft and non-rafts fractions. Western blotting and protein detection were performed as described before, and blots were stained for CD20 (35 kDa), caspase-8 (55 kDa) and FADD (28 kDa), the specific raft marker src-kinase Lyn (56 kDa) and the housekeeping protein Actin (36 kDa).

Discussion

Rituximab sensitizes malignant B-cells to several chemotherapeutic drugs via intracellular mechanisms influencing the pro- and anti-apoptotic balance within a B-cell. Recently, Vega *et al* reported that rituximab is capable of inducing sensitization to apoptosis induced by the death receptor Fas via increased Fas expression and concurrent inhibition of the NF-κB signaling pathway (7,26). In this study, we describe another mechanism involved in the sensitization of lymphoma B-cells to Fas-induced apoptosis. Treatment with rituximab resulted in lateral translocation and clustering of CD20 and Fas at the membrane in raft-like microdomains, resulting in the formation of a functional death inducing signaling complex and thus functional sensitization toward Fas-induced apoptosis induction. Rituximab has been described to cause modification of raft organization on the surface of B cells (37-39). Also, binding by anti-CD20 Abs leads to CD20-multimerization and translocation of CD20 to lipid rafts where it can associate with other signaling molecules (10,40). We hypothesized that rituximab-induced raft-reorganisation facilitates clustering of multimerized death receptors, thereby stimulating the recruitment and activation of caspase-8, resulting in death receptor signaling. A direct interaction between CD20 and death receptors or the death receptor pathway has, to our knowledge, not been described before.

The decision to respond to Fas induced apoptosis was determined within the first two hours of treatment. We therefore focused on the early activation of caspases in the classical induction of apoptosis via rituximab crosslinking, to examine which cell death-inducing processes are initiated by CD20 stimulation. We show that the death receptor pathway plays a significant initiating role in CD20-mediated apoptosis. Although the involvement of caspase-8 activation in rituximab-mediated apoptosis has been described before, until recently this was generally believed to be a secondary result of activation of the mitochondrial pathway (21,40,41). We show that caspase-8 is activated within 2 h of rituximab cross-linking. This is accompanied by the activation of caspase-9, followed by PARP cleavage. Rituximab-induced apoptosis was inhibited by ectopic FLIP-overexpression and by zIETD-FMK, demonstrating rituximab-induced apoptosis to be dependent on caspase-8 activation. This also further underlines that rituximab-induced apoptosis by itself is, at least partly, dependent on activation of the death receptor pathway.

Intertwining of rituximab-induced apoptosis with the functional activation of the death receptor pathway and its consequence for sensitization to anti-Fas-induced apoptosis induction was subsequently demonstrated at the molecular level using immune precipitation and fluorescence microscopy. We show that rituximab cross-

linking leads to membrane clustering of Fas, resulting in recruitment of FADD and caspase-8 to the DISC. To exclude the possibility that rituximab-mediated recruitment of caspase-8 and FADD to the DISC was the result of a secondary apoptotic signaling loop, initiated or amplified by the intrinsic/mitochondrial activation of caspase-9 and/or -3, we performed additional DISC immune precipitation experiments in Ramos cells overexpressing XIAP. XIAP acts as a potent inhibitor of proteolytically processed caspase-9 and -3, preventing downstream continuation of apoptosis signaling as well as secondary triggering of upstream caspases, such as caspase-8 (41,42). In Ramos-XIAP cells, rituximab-induced formation of the DISC and upstream hallmarks of apoptosis (loss of mitochondrial transmembrane potential) were completely intact, confirming that the initiation of the death receptor pathway was a primary consequence of Fas redistribution into multimeric clusters and not a secondary effect of caspase-9 or caspase-3 activation.

The clustering of Fas in rafts upon rituximab treatment in combination with the rituximab-induced increase in expression of Fas described by Vega *et al* (7) may contribute to an efficient DISC formation. Subsequently, caspase-8 is activated, providing an essential step for the execution of apoptosis. The dependence on adequate levels of activated caspase-8 may explain the typical divergence in the kinetics of progression of apoptosis observed after incubation with anti-Fas Abs alone vs the combined treatment of rituximab and anti-Fas Abs (Fig 2C). Possibly, anti-Fas Abs alone do not induce sufficient activation of caspase-8 for persistent induction of apoptosis. Co-treatment with rituximab could provide B cells with an essential signal for enhanced Fas expression and clustering, resulting in an optimized condition for DISC formation, amplification of caspase-8 activation, and, subsequently, to a sustained intracellular delivery of the apoptotic signal. Based on the use of PP2 (src tyrosine kinase inhibitor) and calpeptin (inhibitor of calcium dependent proteases), which did not affect the intrinsic sensitivity toward agonistic anti-Fas Abs or the sensitization of the cells to anti-Fas-induced apoptosis by rituximab (data not shown), signaling via src kinases and calcium does not seem to be involved in the here described sensitization of B cells to Fas-induced apoptosis by rituximab.

Within the Fas signaling cascade, it is not uncommon that clustering of Fas molecules, induced by either Fas overexpression or chemotherapy, can trigger activation of the death receptor pathway independent of Fas ligation by FasL (43). For example, during clonal selection in the germinal center, B cells possess preassembled Fas-DISC, which enables ligand independent induction of apoptosis through Fas (29). Fas trimerization alone, however, is not sufficient for induction and execution of apoptosis. Translocation of Fas to rafts, Fas clustering, and the

subsequent formation of micro-aggregates all appear critical in the regulation of this process (38,39,43).

Vega *et al* (7) described sensitization of B-cells to Fas by rituximab via a pathway that involves inhibition of p38 MAPK and the NF-κB signaling pathway, resulting in the inhibition of the transcription repressor Yin-Yang 1 (YY1) and subsequent upregulation of Fas expression by B-cells (33). In addition to this, we postulate here a mechanism for Fas sensitization by rituximab based on a CD20 stimulation-induced translocation of Fas into lipid rafts, followed by the recruitment of caspase-8 and FADD to raft domains and formation of the DISC. Although forced overexpression of IκBα in Ramos cells did not affect the intrinsic sensitivity toward agonistic anti-Fas Abs or sensitization to anti-Fas-induced apoptosis by rituximab (data not shown), we did observe an increase in Fas expression following treatment of Ramos cells with rituximab, which is in concordance with the results published by Vega *et al*. (7).

It is very well possible that the here described mechanism for sensitization of lymphoma B-cells to anti-Fas Abs by rituximab is not limited uniquely to Fas. A similar phenomenon may occur for other ligands of the death receptor family or for unrelated apoptosis-inducing agents. The capacity of rituximab to influence the membrane composition of B cells by inducing the translocation of other proteins into rafts might be an intrinsic feature of CD20, activated by this Ab, thereby inducing an efficient pro-apoptotic shift in the apoptotic balance of malignant B-cells. Besides in Ramos cells, rituximab-mediated sensitization to anti-Fas-induced apoptosis was observed in Raji cells and BJAB cells, confirming the here described phenomenon to apply more broadly for lymphoma cells. However, results were less clear in primary lymphoma cells, which was due to a high threshold for experimental induction of apoptosis in general (data not shown). The here described findings extend the clinical options for anti-lymphoma therapies combining rituximab with apoptosis inducing agents, targeting either the mitochondrial pathway (e.g., chemotherapy), the death receptor pathway (e.g., FasL or TRAIL), or both. The rationalized implementation of rituximab and CD20 as key regulators of B-cell apoptosis could significantly improve rituximab-mediated treatment strategies for CD20-positive B-cell malignancies in the upcoming years.

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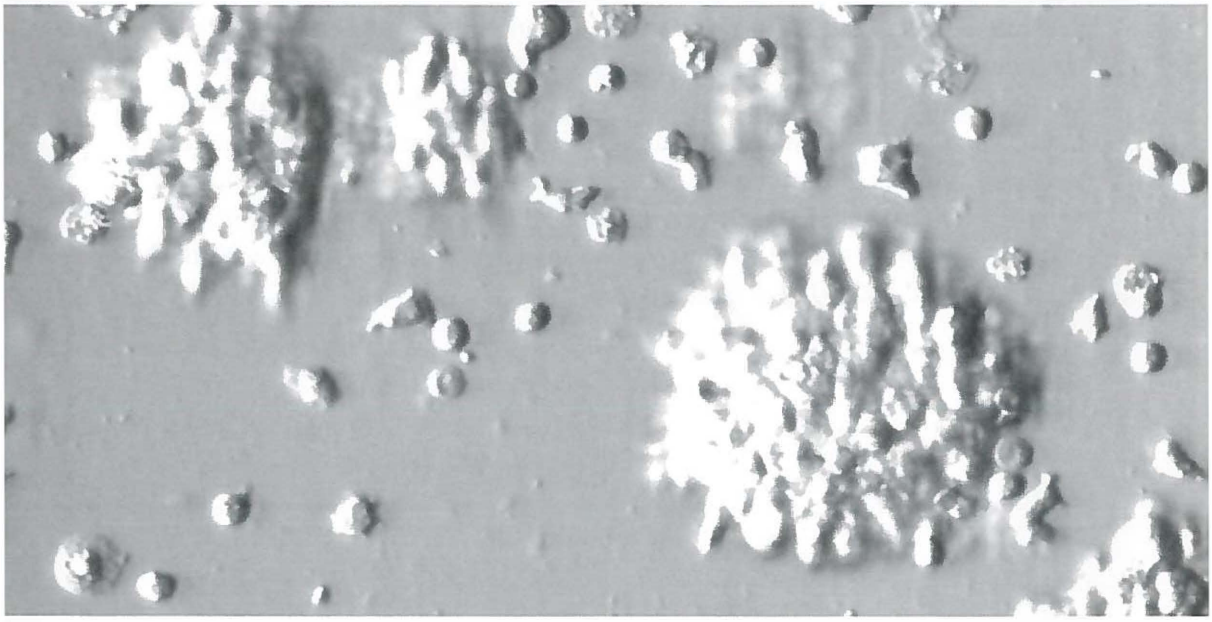
Chapter 5



Physical and functional intertwining of CD20 and HLA-DR signaling pathways.

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Abstract

Antibodies against CD20 are successfully used to treat B-cell malignancies and various autoimmune disorders. Up to now, the physiological role of the CD20 molecule has not been elucidated. CD20 physically interacts with HLA-DR on B-cells, and both induce common signaling pathways. We hypothesized that CD20 plays a direct role in the activation of the B-cell via the HLA-DR/antigen presentation route.

We examined and compared signaling capacities of both CD20 and HLA-DR. Using Ramos cells, we showed that CD20 and HLA-DR share important cascades in B-cell activation, by assessment of apoptosis, calcium conductance, Fas-sensitization, lipid raft formation and cytoskeleton rearrangements. Moreover, we demonstrated functional interaction between CD20 and HLA-DR using the HLA-DR-negative BLS-2 cell line, which was insensitive for rituximab-induced apoptosis, despite abundant CD20 expression. Restoring HLA-DR expression re-introduced sensitivity to CD20-mediated apoptosis. Furthermore, HLA-DR stimulation could induce CD20 raft translocation in Ramos cells, independent of CD20 triggering. We suggest that CD20 may facilitate HLA-DR transmembrane signaling to allow efficient activation of the B-cell via HLA-DR/T-cell receptor interaction.

Introduction

To date, the chimeric anti-CD20 Ab rituximab is the most successful and widest used immunotherapeutic agent in the world. Used as a first line treatment for B-cell non-Hodgkin's lymphoma, mainly in combination with chemotherapy, it induces potent B-cell depletion and results in markedly improved response rates, progression free survival and overall survival in the patients treated (1). Although there have been many speculations on the possible physiological function of CD20, its precise biological role still remains obscure. CD20 is expressed on mature B cells and most malignant B cells, but not on stem cells or plasma cells and it is known to be associated with B-cell development and proliferation (2). Interestingly, no specific ligand for CD20 has been described so far. Using antibodies directed against CD20, an array of cell biological events have been described to occur including CD20 translocation to lipid raft domains and calcium mobilization in B-cells (3-5).

CD20 is a member of the membrane-spanning four-domain subfamily A (MS4A-family), among which are the hematopoietic-cell-specific protein HTm4 and the high affinity IgE receptor beta-chain (FcεRIβ). This family of proteins shares great structure and amino acid homology and all members are likely to be components of oligomeric cell surface complexes involved in signal transduction (6,7). For example, IgE receptor signaling initiated by alpha-chain/ligand interaction, is conveyed to the beta subunit, which functions as an amplifier of FcεRI-mediated cell activation (8). In parallel, such an oligomeric interaction on B-cells may be initiated or regulated by CD20, bypassing the need for CD20 to have a direct ligand-receptor interaction. CD20 has been described to be part of a larger supramolecular complex associated with HLA-DR on B-cells (9,10). Upon Ab stimulation of CD20 and HLA-DR a number of similar cell-biological features are initiated. These include association of CD20 and HLA-DR with lipid rafts (5,11), changes in calcium conductance (12,13) and apoptosis induction (14,15). Stimulation of CD20 as well as HLA-DR activates Src family of tyrosine kinases with downstream activation of phospholipase Cγ (PLCγ) and regulates mitogen-activated protein kinases (MAPKs), such as ERK1/2 and p38MAPK (5,13,16). Leveille *et al* have indicated that CD20 can couple HLA-DR to members of the Src family of tyrosine kinases via an unknown 75-80 kDa protein that directs HLA-DR to protein tyrosine kinase (PTK) activation (10,13). Recently Ivanov *et al* described that two clinically relevant mAbs targeting CD20 and HLA-DR (tositumomab and L243) induce interrelated homotypic adhesion and cell death, dependent on redistribution of actin filaments (17).

The similarities in the cellular responses to CD20 and HLA-DR triggering, and the fact that other members of the MSA4 family can pass on and amplify transmembrane signals without ligand interaction, led us to hypothesize that CD20 may have a ligand-independent supporting function in MHC class II activation in B-cells. In this study we show that HLA-DR and CD20 signaling follow similar kinetics and characteristics and we provide evidence that HLA-DR and CD20-induced apoptosis starts via a common intertwined route involving recruitment in rafts, Src-kinase and caspase-8 activation. Furthermore we show functional interaction between CD20 and HLA-DR and we demonstrate that both HLA-DR and CD20 induce similar cytoskeletal effects and require the release of anchored membrane proteins from the cytoskeleton, which enables each molecule to traffic along the membrane and relocate to functional multireceptor complexes. We suggest a biological role for CD20 in the activation of B-cells by forming a stable platform for HLA-DR molecules to interact with the T-cell receptor and to facilitate specific HLA-DR signaling.

Materials and Methods

Cells and culture media.

The Burkitt's lymphoma type, germinal center derived, B-cell line Ramos-RA1 (ATCC) and its transduced derivate Ramos-EzTD were cultured in RPMI 1640 culture medium (containing 25 mM Hepes and L-glutamin) obtained from Bio-Whittaker (Verviers, Belgium) supplemented with 14% heat-inactivated fetal calf serum (FCS) from Bodinco BV (Alkmaar, The Netherlands), 1 mM sodium pyruvate, 2 mM L-glutamin, 0.5 mM β-mercaptoethanol and 0.1 mg/ml gentamycin sulphate obtained from Bio-Whittaker and 0.02 µg/ml fungizone (Bristol-Meyers, Woerden, The Netherlands). Ramos-EzTD culture medium was additionally supplemented with 1 mg/ml G418 (geneticin). Ramos-EzTD, was kindly provided by Dr. A. deFranco and Dr. N. Gupta (Department of Microbiology and Immunology, University of California, San Fransisco, California, USA) (30). The MHC class II deficient, bare lymphocyte syndrome patient cell line (BLS-2) and its transfected derivates BLS-2 EboSfi (empty vector) and BLS-2 EbosSfi-CIITA (restored wildtype CIITA-cDNA) have been described before and were a kind gift from Dr. P.J. van den Elsen (Leiden University Medical Center, Leiden, the Netherlands) (28). These cells were cultured in RPMI 1640 culture medium, as described above, and transfectants were selected for Hygromycin resistance using 50 µg/ml Hygromycin B (Invitrogen, Breda, The Netherlands). All cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Antibodies and reagents.

Rituximab was kindly provided by Roche Nederland BV (Woerden, The Netherlands). The mouse-anti-human HLA-DR Ab clone BRA30 (HLA-DR Ab) was a gift from IQ Products (Groningen, The Netherlands). F(ab')2-fragments of goat-anti-human (GaH) IgG or goat-anti-human (GaM) IgG used for crosslinking of rituximab or HLA-DR Abs were obtained from Jackson Laboratories (West Grove, PA, USA) as well as the anti-B-cell receptor Ab (anti-BcR-Ab; goat-anti-human IgM). PE-conjugated HLA-DR Abs and FITC-conjugated CD20 Abs were purchased from IQ Products. The cholera toxin B biotin conjugate was obtained from Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands) and Streptavidin-Cy5 from Invitrogen (Breda, The Netherlands). The rabbit-anti-human Phospho-Src Family (Tyr416) Abs were purchased from Cell Signaling Technology, Inc (Beverly, MA, USA). The mouse-anti-human CD20 Abs (L26) and horseradish peroxidase (HRP)-conjugated secondary Abs for Western blotting were obtained from DAKO (Glostrup, Denmark) or, when rabbit Abs were used, from Santa Cruz Biotechnology, Inc. (Tebu-Bio, Heerhugowaard, The Netherlands).The rabbit-anti-human PARP Abs were also obtained from Santa Cruz. Soluble human recombinant Super*Killer*TRAIL was

purchased from Alexis (Kordia BV. Leiden, The Netherlands). The mouse IgM anti-human Fas antibody 7c11 was purchased from Immunotech (Beckman Coulter, Mijdrecht, The Netherlands). The caspase-inhibitors zVAD-FMK (general caspase-inhibitor) and zIETD-FMK (inhibitor of caspase-8) were obtained from Calbiochem (Omnilabo International, Breda, The Netherlands). Triton X-100, propidium iodide (PI), RNAse-A, Puromycin, PMSF, lysophosphatidylcholine and Tween-20 were obtained from Sigma-Aldrich Chemie BV. Poly-acrylamide and Bradford reagent were purchased from Bio-Rad (Hercules, CA, USA). Skim milk was obtained from Difco (BD Biosciences, Alphen aan den Rijn, The Netherlands). Complete protease inhibitors cocktail was purchased from Roche Diagnostics Nederland BV (Almere, The Netherlands).

Induction of apoptosis by crosslinking rituximab or anti-HLA-DR Abs.
One day prior to each experiment, Ramos cells, BLS-2 cells or derivates were harvested and diluted to a concentration of $0.25x10^6$ cells/ml. The following day, cells were harvested and incubated for 30 minutes with 5 µg/ml rituximab or 5 µg/ml HLA-DR Abs at a concentration of $5.0x10^6$ cells/ml in culture medium at 37°C. Hereafter, the cells were washed to remove unbound Abs and plated at a concentration of $1.0x10^6$ cells/ml in 24-well plates. Crosslinking was achieved by adding GaH or GaM in a final concentration of 5 µg/ml. To inhibit caspase activity 20 µM zVAD-FMK or zIETD-FMK was added in the experiments. To examine the dependence on calcium conductance, 3 mM of the calcium chelator EGTA (Sigma-Aldrich Chemie BV) was applied to Ramos cells for 30 minutes reducing free calcium availability. After this pre-incubation period, cells were treated with either rituximab or HLA-DR Abs as described above.

Detection of mitochondrial transmembrane potential.
Apoptosis was determined by detecting loss of mitochondrial transmembrane potential, using DioC6 38. Following treatment of cells as indicated, cells were washed and labeled with DiOC6 (0.1 µM) for 25 min at 37 °C. After labeling, cells were washed and analyzed directly by flow cytometry using excitation and emission wavelengths of 495 and 525 nm, respectively.

DNA fragmentation analysis
Cells ($0.5x10^6$/2ml) were treated as described. After treatment, cells were pelleted and resuspended in 2 ml of ice-cold 70% ethanol and incubated for one hour at 4 °C. After incubation, cells were washed twice with PBS and resuspended in PBS containing RNase (final concentration 0.5 mg/ml) and incubated for 30 minutes at 37°C. Finally, propidium iodide was added at a final concentration of 50 mg/ml and analyzed by flow cytometry.

Preparation of cell lysates for Western blotting.

Cells were lysed in lysis buffer (30 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 10% glycerol, 1mM PMSF, supplemented with complete protease inhibitors cocktail) for 15 minutes at 4°C. Cell debris was pelleted by centrifugation (10 000xg; 4°C) and the supernatant was collected. DNA was sheared by five seconds of sonification on ice (Bandelin sonopuls, Berlin, Germany). The protein concentration of each sample was measured using the Bradford method (39). Western blot samples were prepared by mixing with an equal volume of 2x sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-β-mercaptoethanol, 0.002% bromophenol blue) and heated for five minutes at 95°C. SDS-poly-acrylamide gels were prepared and run according instructions accompanying the Bio-Rad Mini-Protean 3 system. Size-separated protein was transferred onto Bio-Rad Trans-blot nitrocellulose membrane for immunodetection according the manufacturer's protocol.

Protein detection.

Nitrocellulose membrane blots were blocked for at least one hour at room temperature in blocking buffer (PBS, 5% skim milk, 0.1 % Tween-20). Primary antibodies were diluted in blocking buffer as recommended by the supplier and blots were incubated for at least one hour. Subsequently, the blots were washed in PBS containing 0.1% Tween-20 and secondary antibody (HRP-conjugated, 1:2000 diluted in blocking buffer) was added for 30 minutes. Finally, after washing, the conjugates were visualized using Lumi-Light^{PLUS} Western blot substrate (Roche Diagnostics) and Kodak Biomax MR-1 film (Sigma-Aldrich Chemie BV).

Detection of HLA-DR and CD20 by immunofluorescence microscopy.

To perform fluorescent staining of CD20, rituximab was labeled with tetramethylrhodamine (TRITC) according to the manufacturer's protocol (Molecular Probes, Invitrogen, Breda, The Netherlands). Ramos cells ($1.0x10^6$ cells/ml) were cultured for 15 minutes in presence or absence of TRITC-labeled rituximab (5µg/ml) or HLA-DR Abs (5µg/ml), washed using cold PBS and fixed with 3.5% paraformaldehyde (Merck BV, Darmstadt, Germany) during 10 minutes on ice. Samples were subsequently incubated on ice with anti-GM1 cholera toxin B-Biotin, rituximab-TRITC or HLA-DR Abs and the secondary Abs Streptavidin-Cy5 and Qdot 525 goat $F(ab')_2$ anti-mouse IgG conjugate (Quantum Dot Corporation, Hayward, CA, USA), each for 20 minutes after which two rounds of washing with cold PBS were performed. Controls for aspecific staining were performded (pictures not shown). Untreated cells were directly fixed with paraformaldehyde and subsequently stained with anti-GM1 cholera toxin B-Biotin and HLA-DR Abs plus secondary Abs and rituximab-TRITC. After staining cytospots were prepared and

analyzed on a Quantimet 600S digital analysis system (Leica Microsystems Nederland BV, Rijswijk, The Netherlands).

Isolation of lipid rafts by sucrose-gradient ultracentrifugation

Ramos cells ($5.0x10^6$ cells/ml) were incubated with 2 µg/ml rituximab or HLA-DR Abs for 15 minutes in culture medium at room temperature. After incubation, medium was removed by centrifugation and the cells were resuspended in ice-cold Tris-buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA) with 1% Triton X-100 and 1 µM aprotinin, leupeptin, and pepstatin A, all obtained from Sigma-Aldrich Chemie BV. The suspension was vortexed and kept on ice for 30 minutes. The suspension was homogenized by twenty strokes through a 21-gauge needle. To isolate the lipid raft fraction, lysates were mixed with an equal volume of ice cold 80% sucrose in Tris-buffer and overlaid with a 35% sucrose and a 5% sucrose layer in Tris-buffer. The gradients were centrifuged in a Beckman SW41 swing-out rotor at 40,000 rpm for 18 hours at 4°C, to allow equilibration. After centrifugation, 10 fractions were taken from top to bottom of the gradients. The fractions were transferred to micro test tubes and vortexed. Protein content of the fractions was determined and 10 µg of protein was taken from each fraction and adjusted to 10 µg/ml. 5 µL of sodium deoxycholate 25 mg/ml was added to each tube, followed by 5 minutes incubation on ice. Subsequently, 60 µL of trichloroacetic acid 100% (w/v) was added and the samples were vortexed, incubated on ice for 10 minutes and centrifuged for 15 minutes, 4500 *g* at 4°C. The supernatant was removed and the precipitated proteins were dissolved in 20 µL sample buffer, neutralized for 10 minutes with ammonia vapor and stored at -20°C. Western blotting and protein detection were performed as described before.

Detection of HLA-DR and CD20 by flow cytometry

To detect HLA-DR or CD20 expression on Ramos, BLS-2, BLS-2 $^{\text{empty vector}}$, BLS-2 $^{\text{CIITA}}$ cells, PE- labeled HLA-DR or FITC-labeled CD20 Abs in saturating concentrations were coated to the cells for 30 minutes on ice. Unbound Ab was removed by washing and cells were assessed by FACS analysis.

F-actin polymerization

Ramos cells ($1.0x10^6$cells/ml) were incubated for the indicated time periods with rituximab, HLA-DR Abs or control IgG Abs. After stimulation, cytoskeleton-remodeling was interrupted by incubation with an equal volume of fixation buffer (7% paraformaldehyde and 0.2 mg/ml lysophosphatidylcholine in PBS) for 10 minutes on ice. After fixation and permeabilization, F-actin was labeled with phalloidin (0.2 units/ml Oregon Green 488 phalloidin (Molecular Probes, Invitrogen)) by incubating the cells under dark conditions, for 30 minutes at room

temperature. F-actin staining was determined by flow cytometry directly following the incubation. Relative F-actin polymerization levels are expressed by correcting the mean fluorescence intensity (MFI) of stimulated Ramos cells for the MFI of unstimulated cells (MFI stimulated cells / MFI unstimulated cells).

Statistics

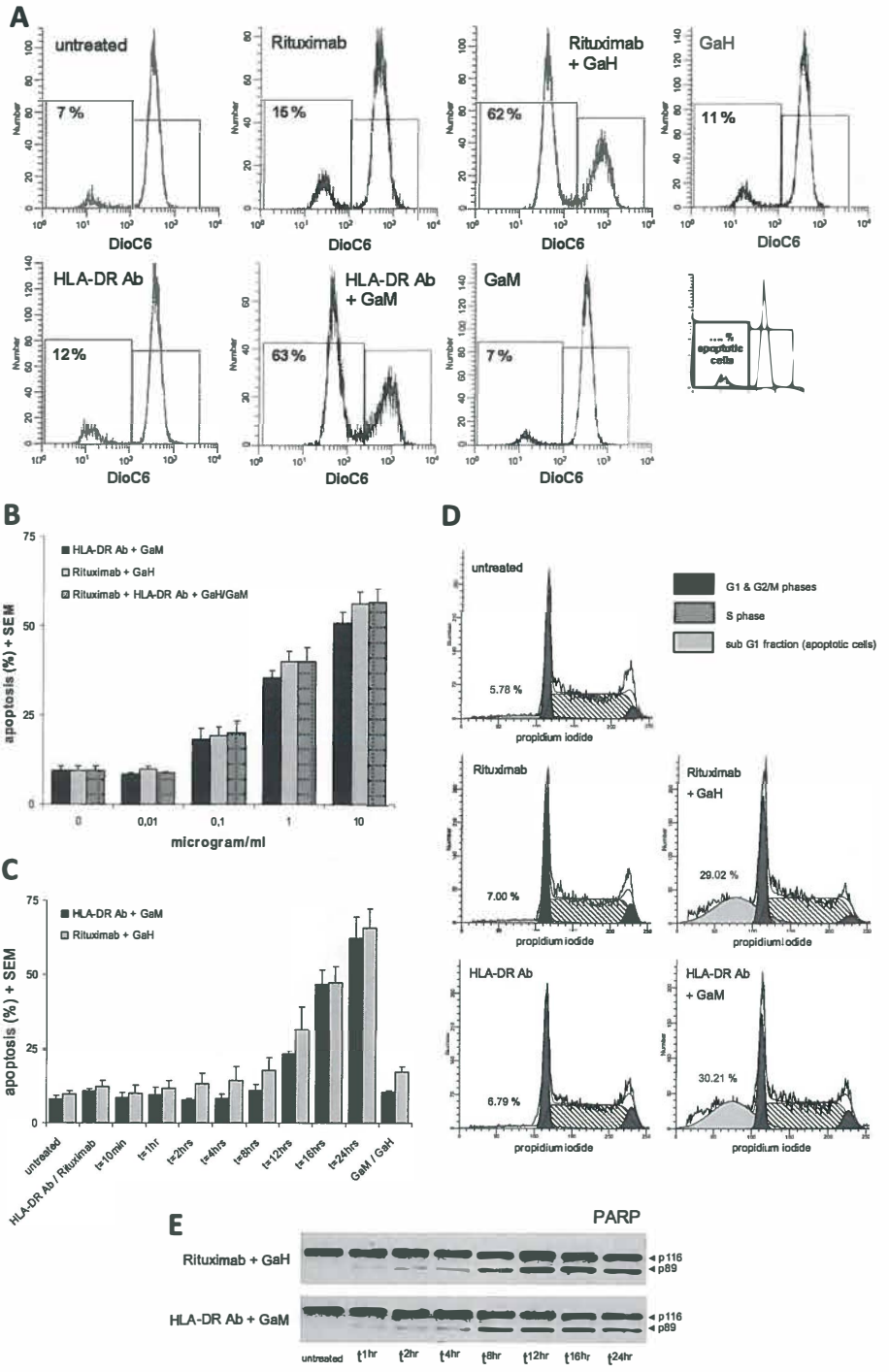
All described experiments were performed at least in quadruplicates and results are depicted representing the means of the data and the SEM for all applicable experiments. All fluorescent or western blot pictures were representatives of multiple experiments performed on different days

Results

Rituximab and HLA-DR Abs induce apoptosis following similar kinetics.

To investigate the apoptotic cascade induced by rituximab or anti-HLA-DR antibody (further referred to as HLA-DR Ab) crosslinking, Ramos cells were treated with different concentrations of the Abs, followed by matching concentrations of secondary Ab. To exclude aspecific, Fc-receptor mediated effects, isotype control experiments were performed for all experiments described in this study. No aspecific effects were detected (data not shown). Apoptosis was determined at the level of mitochondrial transmembrane potential loss, using DioC6 staining followed by flow cytometrical analysis (Fig 1A). A dose dependent induction of apoptosis was noted using either rituximab or HLA-DR Ab. The combination of crosslinked rituximab and anti-HLA-DR Abs did not augment apoptosis at any given concentration compared to the percentage of apoptosis detected by either one of the crosslinked Abs applied alone (Fig 1B). This suggested a common and proximal converging pathway of apoptosis induction, which was the subject of further investigation. To this end we determined the kinetics of apoptosis induction by rituximab and HLA-DR Abs defined by the loss of mitochondrial transmembrane potential. Apoptosis induced by both Abs displayed similar kinetics and was first detected after approximately eight hours of incubation, increasing up to 65% after 24 hours (Fig 1C). These findings were confirmed by DNA-fragmentation analysis, using PI staining (Fig 1D) and Western-blot analysis, by assessing poly (ADP-ribose) polymerase (PARP) cleavage after rituximab or HLA-DR Ab crosslinking (Fig 1E).

Figure 1. (right page) HLA-DR Abs and rituximab induce apoptosis following similar kinetics. (A) Ramos cells ($1.0x10^6$/ml) were incubated with 5 µg/ml HLA-DR Ab, rituximab or a combination of both for 30 minutes, washed, and subsequently incubated over night (16 hours). Apoptosis was quantified by detecting mitochondrial membrane potential loss, determined by DioC6 staining. (B) Ramos cells ($1.0x10^6$/ml) were incubated with different concentrations of HLA-DR Ab, rituximab or a combination of both for 30 minutes, washed, and subsequently incubated over night (16 hours) with corresponding amounts (0.01, 0.1, 1.0 and 10 µg/ml) of crosslinking Abs (GaM and GaH). Apoptosis was quantified by detecting mitochondrial membrane potential loss, determined by DioC6 staining. The mean result of quadruplicate assays along with the SEM is displayed for each condition. (C) Ramos cells ($1.0x10^6$/ml) were incubated with 5 µg/ml HLA-DR Ab or rituximab for 30 minutes, washed, and subsequently incubated with 5 µg/ml GaM or GaH for the indicated time periods, increasing from 10 minutes up to 24 hours. Apoptosis was determined by DioC6 staining and the mean result of quadruplicate assays is displayed for each incubation period along with the SEM. (D) Apoptosis was confirmed by incubating Ramos cells as described above with 5 µg/ml HLA-DR Ab or rituximab, where indicated followed by incubation with GaM or GaH (5 µg/ml) for 16 hours. After the incubation period, apoptosis was determined by detecting DNA fragmentation by PI staining. (E) Western-blots were prepared from sample lysates matching the indicated time samples from Fig 1C and stained for PARP cleavage (116 kDa and 89 kDa). The depicted blots are representatives of three separate experiments.


A
untreated
7 %
Rituximab
15 %
Rituximab + GaH
62 %
GaH
11 %
HLA-DR Ab
12%
HLA-DR Ab + GaM
63 %
GaM
7 %
... % apoptotic cells
Number
DioC6
B
HLA-DR Ab + GaM
Rituximab + GaH
Rituximab + HLA-DR Ab + GaH/GaM
apoptosis (%) + SEM
microgram/ml
C
HLA-DR Ab + GaM
Rituximab + GaH
apoptosis (%) + SEM
untreated
HLA-DR Ab / Rituximab
t=10min
t=1hr
t=2hrs
t=4hrs
t=8hrs
t=12hrs
t=16hrs
t=24hrs
GaM / GaH
D
untreated
5.78 %
G1 & G2/M phases
S phase
sub G1 fraction (apoptotic cells)
propidium iodide
Rituximab
7.00 %
Rituximab + GaH
29.02 %
HLA-DR Ab
6.79 %
HLA-DR Ab + GaM
30.21 %
E
PARP
Rituximab + GaH
HLA-DR Ab + GaM
p116
p89
untreated
t1hr
t2hr
t4hr
t8hr
t12hr
t16hr
t24hr

Both rituximab and HLA-DR Ab mediated apoptosis is dependent on calcium mobilization.

Both CD20 and HLA-DR are described to affect calcium mobilization in B-cells upon stimulation (12,13). To investigate whether apoptosis, induced by rituximab and HLA-DR Abs, is dependent on calcium mobilization, Ramos cells were pre-treated with EGTA to remove all available free calcium, and subsequently were incubated over night with rituximab and HLA-DR Abs. As controls, Abs activating the B-cell receptor (IgM) or soluble TRAIL (TRAIL) were applied, either with or without EGTA pre-treatment. BcR activation and apoptosis induction is calcium dependent, whereas apoptosis induction by TNF family death receptor members (e.g. TRAIL) is calcium independent (13,18,19). As shown in Fig 2, apoptosis induced by rituximab as well as by HLA-DR Ab crosslinking was significantly inhibited by EGTA, suggesting a common, calcium dependent mechanism. In line with the results described previously by others, EGTA did inhibit IgM-induced apoptosis, whereas TRAIL-induced cell death was not affected by EGTA.

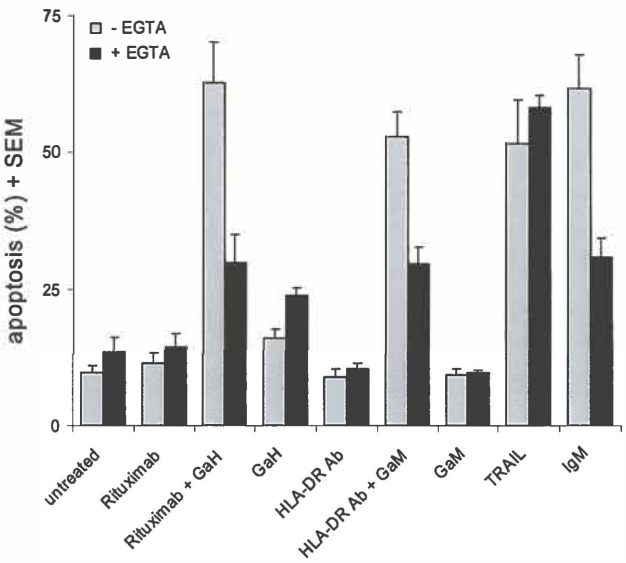


Figure 2. HLA-DR Ab and rituximab mediated apoptosis depends on calcium mobilization. Ramos cells ($1.0x10^6$/ml) were pretreated, where indicated, with 3 mM EGTA and subsequently incubated, as described above, with 5 μg/ml HLA-DR Ab or rituximab with or without crosslinking for 16 hours. As a positive control for calcium dependence 5 μg/ml anti-BcR Ab (IgM) was applied. As a negative control for calcium dependence 100 ng/ml soluble TRAIL (TRAIL) was applied. Apoptosis was determined by DioC6 staining and in the graph the mean result of quadruplicate assays is displayed for each incubation period along with the SEM.

Rituximab and HLA-DR Ab induced apoptosis depends on caspase activation.
Apoptosis induced by both rituximab and HLA-DR Ab oligomerization can involve activation of caspases (20,21). To explore similarities in the involvement of effector and initiator caspases in our model, the pan-caspase inhibitor zVAD-FMK and the initiator caspase-8 inhibitor zIETD-FMK were applied. zVAD-FMK completely blocked apoptosis induced by both rituximab and HLA-DR Ab. Partial inhibition was obtained using the caspase-8 inhibitor zIETD-FMK. The residual levels of apoptosis corresponded with either one of both antibodies (Fig 3A). These results suggest a similar involvement of caspases in both rituximab and HLA-DR Ab induced apoptosis.

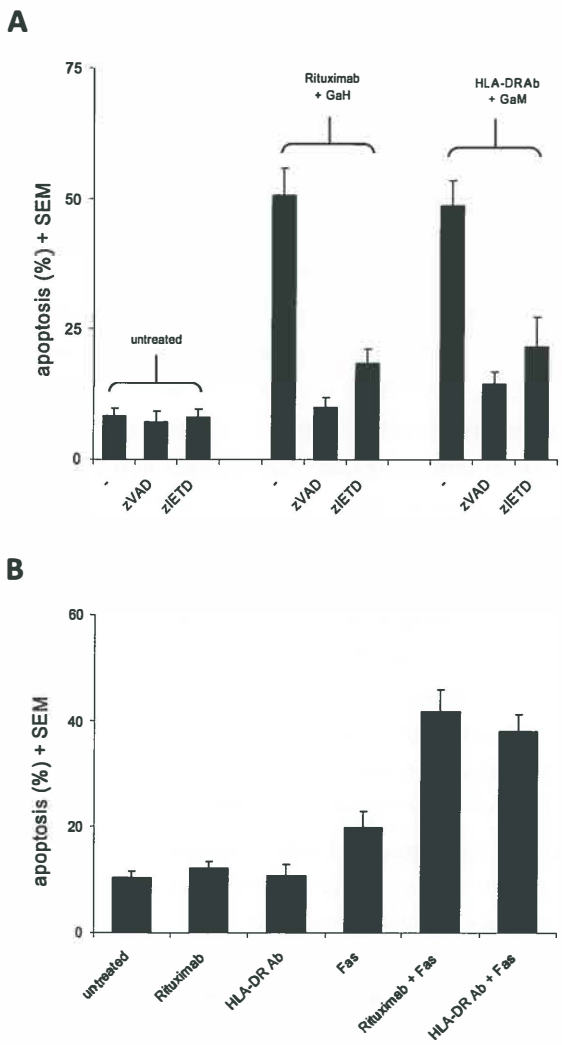


Figure 3. HLA-DR Ab and rituximab induced apoptosis is dependent on the activation of caspases. (A) Apoptosis was induced in Ramos cells as described. Where indicated, the caspase inhibitors zVAD-FMK and zIETD-FMK where added, both during pre-incubation with HLA-DR As or rituximab as during the over night period of crosslinking by GaM or GaH. Apoptosis was determined by DioC6 staining and mean results of quadruplicate assays are depicted along with the SEM. (B) Ramos cells ($1.0x10^6$/ ml) were incubated over night with HLA-DR Ab (5 µg / ml), rituximab (5 µg/ml) or the mouse IgM anti-human Fas antibody 7c11 (Fas 2 µg / ml) alone, or combinations of HLA-DR Ab/Fas and rituximab / Fas in corresponding combinations. Apoptosis was determined by DioC6 staining and mean results of quadruplicate assays along with the SEM are displayed for each condition.

Furthermore, rituximab can sensitize lymphoma cells to Fas-induced apoptosis (22,23), a phenomenon that has also been described for HLA-DR Abs 24. The mechanism causing this sensitization is suggested to involve rituximab or HLA-DR-induced increase in Fas expression, clustering of Fas-receptors in raft-like membrane domains, increased recruitment of FADD (Fas-associated death domain) and caspase-8 to the DISC (death-inducing signaling complex) and subsequent activation of caspase-8 and other downstream caspases. Although Ramos cells have an intrinsic resistance to apoptosis induced by Fas agonistic Abs, this resistance could be overcome by simultaneous treatment of these cells with Fas agonistic Abs plus rituximab or HLA-DR Abs. Without crosslinking, rituximab and HLA-DR Abs did not induce any apoptosis, compared to background levels in untreated cells. The IgM-type anti-Fas Ab 7C11 induced only minor levels of apoptosis as a single agent. However, combining rituximab or HLA-DR Abs with 7C11 showed a synergistic increase in cell death (Fig 3B). This increase in apoptosis induction was similar for both the combination rituximab/7C11 and HLA-DR Ab/7C11, suggesting rituximab and anti-HLA-DR Ab mediated Fas-sensitization to be the result of a common intracellular signaling pathway.

HLA-DR stimulation triggers CD20 translocation into rafts.

Considering the reported physical interaction between CD20 and HLA-DR, we examined the membrane localization of both, following incubation with rituximab or anti-HLA-DR Ab. Without incubation, both CD20 and HLA-DR were present on the membrane in a homogeneous dispersed pattern. Upon incubation with each of both Abs, this pattern became patched, indicating that translocation and clustering of both CD20 and HLA-DR to raft-like domains took place upon CD20 or HLA-DR stimulation (Fig 4A). Interestingly, HLA-DR stimulation not only resulted in translocation of HLA-DR, but also of CD20 to raft-like domains (Fig 4B). Inversely, CD20 stimulation by rituximab did not induce HLA-DR translocation to rafts. Raft translocation was confirmed by co-localization of HLA-DR or CD20 with GM1, a sphingolipid widely used as marker for lipid raft formation (25). Apparently, stimulation of HLA-DR resulted in a functional signal directed to CD20 followed by concomitant translocation of both molecules to rafts. These data suggest that HLA-DR signaling is closely related to and may functionally affect CD20 signaling. Translocation of CD20 and HLA-DR to rafts occurred within minutes after starting incubation and persisted during the first hour. After this, the presence of these molecules in raft-like domains decreased (results not shown).

To confirm the translocation of CD20 into rafts upon HLA-DR stimulation, we incubated Ramos cells with rituximab or anti-HLA-DR Abs and subsequently isolated lipid rafts by sucrose-gradient ultracentrifugation. Translocation of CD20 to

rafts was determined by Western blot analysis. As a control for proper lipid raft isolation, the blots were also probed for the lipid raft marker phospho-src family (Tyr416), which is constitutively present in raft fractions and only minimally in non-raft fractions. In concordance with the fluorescence microscopy data, CD20 was found to transfer into raft fractions upon incubation of Ramos cells with rituximab as well as with anti-HLA-DR Abs (Fig 4C).

Intact MHC class II expression is necessary for rituximab mediated apoptosis.
Fluorescence resonance energy transfer (FRET) experiments confirm the close interaction between HLA-DR and CD20 molecules (26). If CD20 is part of a signaling complex surrounding and depending on MHC class II functionality, disruption of MHC class II expression might affect CD20 signaling capacities. To investigate functional interaction between CD20 and HLA-DR, we used the BLS-2 cell line, derived from a patient suffering from bare lymphocyte syndrome, displaying a deficiency for MHC class II (27). These cells lack functional CIITA (class II transactivator), which is responsible for functional transcription of MHC-class II genes. Wild type CIITA DNA was transfected back into this cell-line to re-initiate MHC-class II expression (28). Cells of all three BLS-2 subtypes (parental, empty vector transfected and CIITA transfected) plus Ramos cells were stained for CD20 and HLA-DR expression. All cell lines expressed CD20, although slightly higher expression was found in the BLS-2 cell types. Both the BLS-2 and the BLS-2 $^{\text{empty vector}}$ cell lines did not express detectable levels of HLA-DR, whereas Ramos and BLS-2 $^{\text{CIITA}}$ did express HLA-DR as detected by flow cytometry (Fig 5A). Although the BLS-2 cell line shows significant CD20 surface expression, rituximab crosslinking did not induce apoptosis in the parental or empty vector cells. Re-introduction of HLA-DR expression, however, restored the sensitivity of BLS-2 lines to rituximab induced apoptosis, suggesting a direct link between the membrane presence of HLA-DR and the induction of efficient CD20 signaling and apoptosis (Fig 5B).

HLA-DR and CD20 signaling pathways require cytoskeletal based remodeling of the membrane.
Based on the known involvement of rafts in CD20 mediated signaling and the intertwined regulation of membrane and cytoskeleton remodeling, we studied the involvement of the cytoskeleton in HLA-DR and CD20 stimulation. Specifically, we measured the levels of F-actin depolymerization in Ramos in time upon stimulation of both CD20 and HLA-DR. As shown in Fig 6A, both HLA-DR and CD20 stimulation led to a similar transient depolymerization of F-actin, providing evidence for a similar form of receptor induced cytoskeletal remodeling via HLA-DR and CD20.

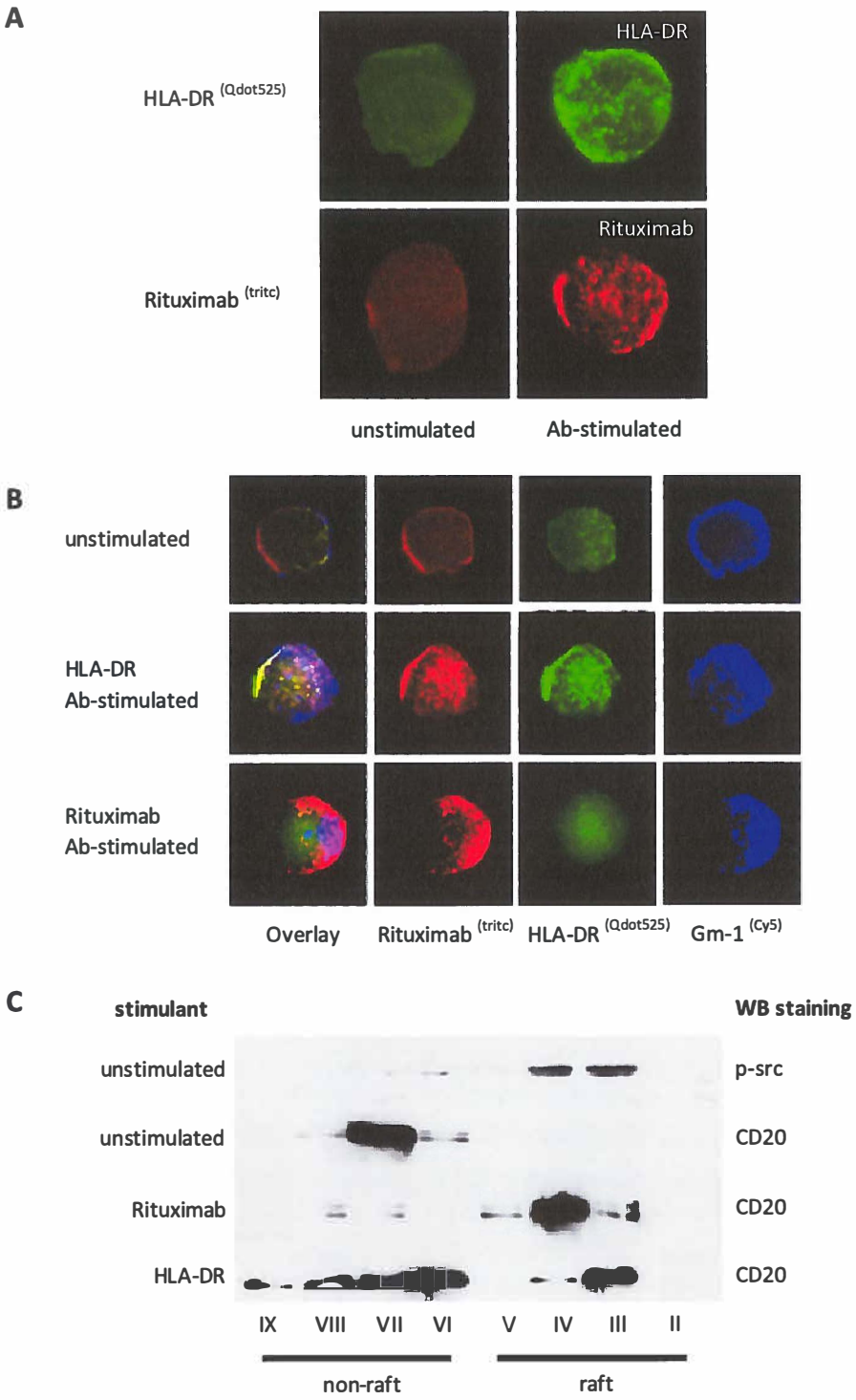

A
HLA-DR (Qdot525)
Rituximab (tritc)
HLA-DR
Rituximab
unstimulated
Ab-stimulated
B
unstimulated
HLA-DR
Ab-stimulated
Rituximab
Ab-stimulated
Overlay
Rituximab (tritc)
HLA-DR (Qdot525)
Gm-1 (Cy5)
C
stimulant
WB staining
unstimulated
unstimulated
Rituximab
HLA-DR
p-src
CD20
CD20
CD20
IX VIII VII VI V IV III II
non-raft
raft

Figure 4. (left page) HLA-DR and CD20 translocate into raft-like domains upon Ab stimulation. (A) Ramos cells ($1.0x10^6$ cells/ml) were stimulated for 15 minutes with TRITC-labeled rituximab (5 µg/ml) or HLA-DR Abs (5 µg/ml) and fixated during 10 minutes on ice. Samples were subsequently incubated on ice with rituximab-TRITC or HLA-DR Abs and the secondary Qdot 525 goat $F(ab')_2$ anti-mouse IgG conjugate each for 20 minutes. Unstimulated cells were directly fixed and subsequently stained with HLA-DR Abs plus secondary Abs or rituximab-TRITC. After staining cytospots were prepared and analyzed. Pictures are representatives of three different experiments (original magnification x 40). (B) Ramos cells were stimulated with HLA-DR Abs or rituximab as described above and both unstimulated and stimulated samples were fixed and incubated with HLA-DR Abs and secondary Qdot 525-conjugated Abs, rituximab-TRITC or GM-1 Abs (cholera toxin B-biotin) and secondary streptavidin-Cy5, conform protocol. Cytospots were analyzed and pictures plus overlays are representatives of three different experiments (original magnification x 40). (C) Ramos cells ($5.0x10^6$ cells/ml) were incubated with 2 µg/ml rituximab or HLA-DR Abs for 15 minutes at room temperature. Lipid raft fractions were isolated from stimulated or unstimulated cells by sucrose-gradient ultracentrifugation and fractions were analysed by western blotting. Western-blots were stained for phosphorylated Src (60 kDa) or CD20 (33 kDa).The depicted blots are representatives of three separate experiments.

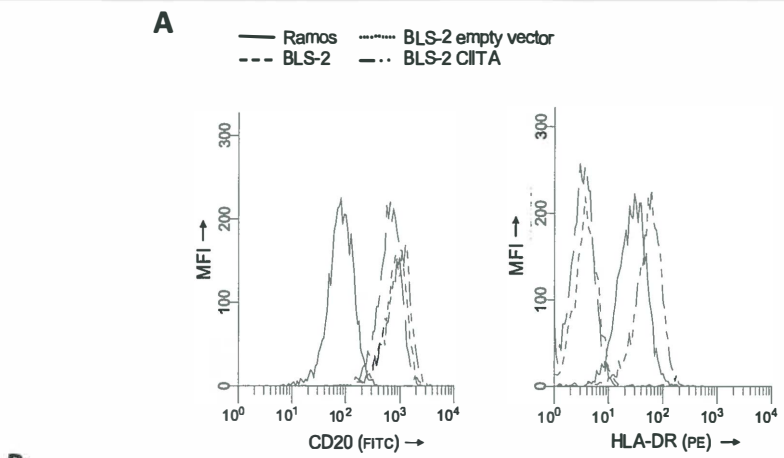


Figure 5. Intact MHC class II expression is necessary for rituximab mediated apoptosis. (A) Ramos, BLS-2, BLS-2 $^{empty\ vector}$, BLS-2 ^{CIITA} cells were incubated for 30 minutes on ice with saturating amounts of PE-labeled HLA-DR Abs or FITC-labeled CD20 Abs and expression was detected by FACS analysis. (B) Apoptosis was induced in BLS-2, BLS-2 $^{empty\ vector}$, BLS-2 ^{CIITA} cells over night as described and detected by DioC6 staining. Mean results of quadruplicate assays are shown along with the SEM.

The IgG control antibody did not affect the polymerization state of F-actin. To further investigate the involvement of lateral membrane dynamics in CD20 and HLA-DR signaling, a transfected Ramos derivate was used, containing a mutated form of ezrin; EzTD. Ezrin is a member of the ERM (ezrin/radixin/moesin) family of proteins, which are described to be structural linkers between plasma membrane proteins and the actin cytoskeleton (29). EzTD contains a phosphomimetic mutation replacing threonine (T) 567 of Ezrin to aspartic acid (D) resulting in a constitutively activated, cytoskeleton bound state. The recombinant protein also contains the transmembrane domain of LAT by which it is constitutively anchored to membrane rafts, preventing lateral remodeling of the plasma membrane (30).Wildtype Ramos and Ramos-EzTD cells were treated with rituximab or HLA-DR Abs, with or without crosslinking and apoptosis was measured by DioC6 staining. Apoptosis was significantly inhibited for both HLA-DR and CD20 triggering in the Ramos-EzTD cells as compared to apoptosis induced within the wildtype Ramos cells (Fig 6B). No differences were seen in CD20 and HLA-DR membrane expression between Ramos and Ramos-EzTD cells. Intrinsic sensitivity to apoptosis in Ramos-EzTD cells is intact since induction of apoptosis by TRAIL commences normally (results not shown).

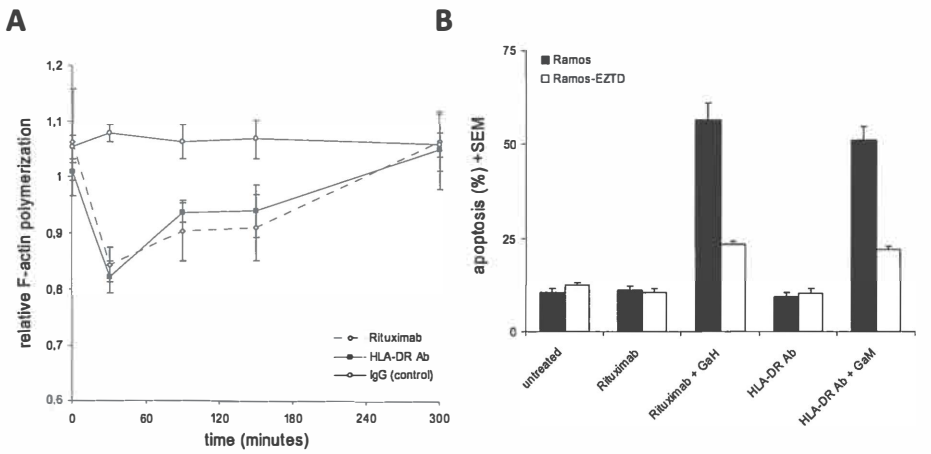


Figure 6. HLA-DR and rituximab signaling pathways require cytoskeletal based remodeling of the membrane. (A) Ramos cells ($1.0x10^6$cells/ml) were incubated for the indicated time periods with rituximab, HLA-DR Abs or control IgG Abs. Cells were fixated and stained for F-actin with Oregon Green 488 phalloidin by incubating the cells for 30 minutes at room temperature. F-actin polymerization was determined by flow cytometry and relative F-actin polymerization was calculated by correcting for F-actin polymerization in untreated cells. Mean results are depicted together with the SEM. (B) Ramos cells or Ramos-EzTD cells were incubated with HLA-DR Abs and rituximab in presence or absence of GaM and GaH as indicated to induce apoptosis conform previous experiments. Apoptosis was determined by DioC6 staining and mean results of quadruplicate assays, together with the SEM, are shown.

Discussion

CD20 and HLA-DR are molecules present on the surface of B-cells that have not been directly associated in function before. In the current study we explored the relation between HLA-DR and CD20 in order to increase our understanding on the biological function of CD20 on B-cells. Given the mechanistic, experimental and conceptual complexity of the signaling events, we have chosen to limit our experiments to one specific B-cell line. We deliberately limited all experiments to one model to provide a solid basis for the observed similarities between CD20 and HLA-DR signaling and to provide evidence that these similarities are indeed due to intrinsic cellular effects and not due to culturing or cell line differences.
We show that signaling and apoptosis induced via CD20 and HLA-DR crosslinking is executed following similar kinetics and characteristics suggesting proximal convergence of the signaling pathways at the level of the receptors itself. In addition, we try to provide evidence for a direct functional interaction between CD20 and HLA-DR in terms of signaling. In a previous report of Tobin *et al*, it was demonstrated that in certain cell lines other than Ramos, combined treatment with rituximab and anti-HLA-DR synergistically induces apoptosis (31). This may relate to a variation in sensitivity for single or crosslinked Ab treatment between different cell models, a phenomenon which has been described previously (15). In concordance with the results shown by Tobin *et al*, we did not observe any synergistic induction of apoptosis in Ramos cells towards both Abs applied simultaneously, irrespective of the concentration of Ab used. This suggests that in Ramos cells CD20 and HLA-DR signaling converge at a proximal level and commence via the same signaling pathway.

Apoptosis, as induced by rituximab or HLA-DR Abs, involves both caspase dependent and independent mechanisms (20,21,32,33). Part of the discrepancy in the degree of dependence on caspase activation found in various reports and between research laboratories might be explained by the use of different Ab clones, cell lines and variation in culture techniques. In our model, both rituximab and HLA-DR Ab induced apoptosis was dependent on caspase activation, as indicated by the ability of the pan-caspase inhibitor zVAD-FMK to inhibit apoptosis induction. Recently, we have demonstrated the dependence of CD20-induced apoptosis on caspase activity and involvement of the death receptor pathway in rituximab-mediated apoptosis (23). Both HLA-DR and CD20 mediated apoptosis appeared to be inhibited by applying the caspase-8 inhibitor zIETD-FMK, indicating involvement of caspase-8. Activation of caspase-8 by CD20 and HLA-DR triggering might explain the sensitization for Fas induced apoptosis we and others observed for both HLA-DR and CD20 stimulation (23,24). We suggest that HLA-DR and CD20

can activate the Fas pathway via a common intertwined mechanism involving increased Fas receptor expression in combination with receptor relocation and clustering at the membrane allowing efficient processing and activation of caspase-8.

In this respect it was relevant to study the effects of CD20 or HLA-DR stimulation on their membrane location. Our results indicated a direct physical and functional effect of HLA-DR stimulation on the localization of CD20 at the cell membrane and signaling via CD20. This was supported by the following observations: firstly, Ab binding to HLA-DR or CD20 induced translocation of the triggered protein towards lipid rafts, secondly HLA-DR stimulation induced an independent clustering of CD20 into a raft-like, patched pattern on the cell membrane, whereas Ab binding to CD20 did not induce HLA-DR clustering. Finally, cells stimulated by HLA-DR Abs were found to shift CD20 from non-raft into raft fractions. Apparently, HLA-DR Ab induced membrane remodeling has an effect on the CD20 protein that is independent of CD20 Ab binding. We suggest that CD20 has a similar function as its family member, the β subunit of FcεRI, which regulates signaling of receptors located in close proximity. This signal regulation function has also been described for tetraspanins (34) which share significant sequence and structural homology with CD20. Like CD20, tetraspanins are characterized by four transmembrane domains in which both the amino- and carboxy-termini are positioned at the cytoplasmic side of the membrane. In addition, tetraspanins are abundantly expressed in cells of the immune system where they orchestrate inter-molecular interactions at the membrane without the need for receptor-ligand interaction. We hypothesize that HLA-DR induced signaling is conveyed intracellularly by CD20.

This functional interrelation of HLA-DR and CD20 was further demonstrated using the HLA-DR negative cell line BLS-2. Despite abundant CD20 expression, this cell line did not respond to CD20 crosslinking. Restoring HLA-DR expression restored the sensitivity of this cell line for rituximab-induced apoptosis suggesting the need for a functional platform created by HLA-DR and other associated proteins to activate intracellular signaling via CD20.

The significance of functional multi-receptor membrane domains to support CD20 signaling was underscored by our studies using membrane domain remodeling deficient Ramos EzTD cells. Translocation of proteins from one membrane compartment to the other is dependent on interactions with the actin cytoskeleton (35). Raft dependent macrostructures like the immune synapse can only be maintained in the fluid membrane environment through anchoring of rafts to the underlying actin cytoskeleton. The association of MHC-class II molecules with the

cytoskeleton plays a critical role during T-cell/APC interaction (36,37). We investigated the effects of HLA-DR or CD20 stimulation on the actin cytoskeleton and noticed a rapid and highly comparably HLA-DR and CD20 stimulation-dependent depolymerization of F-actin which was slowly recovered by repolymerization in time. We hypothesize that F-actin depolymerization enables CD20, HLA-DR and related proteins to translocate towards raft-like domains. Subsequent repolymerization of F-actin induces re-attachment to the cytoskeleton in these rafts, where signaling can be initiated. Ezrin is one of the ERM protein family members, connecting cell surface proteins to the cytoskeleton. Using a constitutively active mutant form of ezrin, the natural lateral plasma membrane dynamics are blocked (30). Within this environment we observed that both rituximab and HLA-DR Ab induced apoptosis was significantly inhibited. Apparently, both HLA-DR and CD20 require the release of anchored membrane proteins from the cytoskeleton, which enables each molecule to traffic along the membrane and relocate to functional multireceptor complexes.

In summary, in this study we investigated the intertwined characteristics of the signaling cascades of HLA-DR and CD20. From our study, we concluded that the signaling cascades started by either molecule are identical, involve physical association and rely on active cytoskeleton mediated membrane remodeling. Our results indicate that CD20 is involved in conveying signals originating from HLA-DR stimulation. It is likely that CD20, like its family members of the MS4A family, is present in a multimolecular membrane complex, interacting with HLA-DR and thereby facilitating HLA-DR complex formation and amplifying transmembrane signaling. The biological function of CD20 may therefore be found within the immune synapse, where CD20 supports the HLA-DR signaling platform and prompts it to efficient antigen presentation.

Acknowledgements

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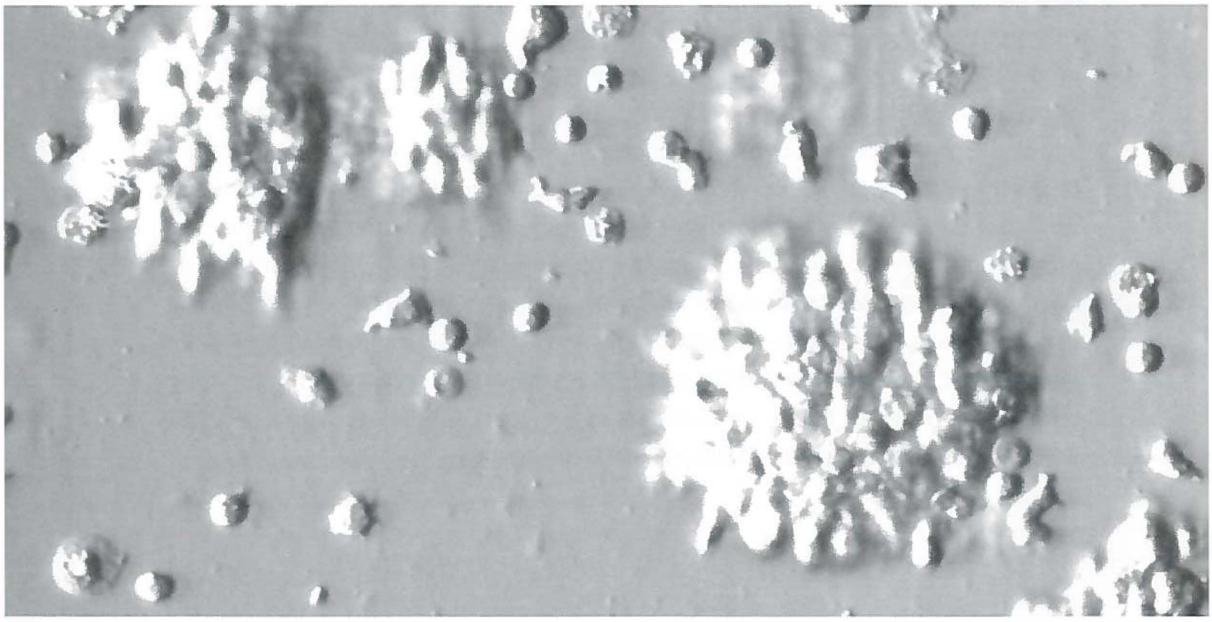
Chapter 6



The anti-leukemia therapeutic effect of BIS20x3 in a NOD/scid mouse model.

In preparation.

Abstract

The bispecific antibody BIS20x3 has a dual specificity for both CD20 and CD3 and effectively directs the lytic potential of cytolytic T-cells towards malignant, CD20-positive B-cell lines. In this study, we demonstrated the *in vitro* and *in vivo* capacity of BIS20x3 to costimulate and activate T-cells towards patient-derived CD20-positive leukemia cells. In *vitro,* BIS20x3 efficiently and specifically opsonized CD20-positive ALL cells and allowed efficient redirected cytotoxicity of CTL, with maximal cytolytic capacity at 0.5 µg/ml BIS20x3. Lysis was CD20 specific and rapid, resulting in up to 70% cell death within four hours of incubation. We showed that BIS20x3 opsonized CD20-positive ALL cells induced efficient T-cell activated *in vitro*, as determined by CD25 expression. T-cell populations expanded and leukemia progression was strongly inhibited in cell culture studies. Subsequently, we introduced BIS20x3 into an *in vivo* NOD/scid mouse model, developed to assess the anti-leukemic efficacy of donor lymphocyte infusion (DLI). In these experiments, BIS20x3 treatment, in combination with DLI resulted in significant lower leukemic cell counts, compared to counts obtained with DLI treatment alone, and progression free survival was increased to almost 80 days. In conclusion: we showed that BIS20x3 can activate and retarget freshly isolated T-cells to patient-derived leukemia cells in an efficient, rapid and highly lytic manner. Upon BIS20x3 binding, the isolated T-cell population can expand and suppress leukemic cell proliferation *in vitro*. Finally, *in vivo* data suggest a promising beneficial effect by BIS20x3 on the treatment of CD20-positive B-lineage ALL.

Introduction

CD20 is an efficient target for antibody (Ab) mediated anti-tumor immunotherapy. Treatment of B-cell lymphoma patients with rituximab, a chimeric IgG1 Ab, induces cell-death in CD20-positive malignant B-cells by means of Ab dependent cellular cytotoxicity (ADCC), complement dependent lysis (CDC) and via CD20-hypercrosslinking (1). Rituximab induces potent B-cell depletion and results in markedly improved response rates, progression free survival and overall survival in the patients treated when used as a first line treatment, mainly in combination with chemotherapy (2,3). However, especially in advanced disease, rituximab treatment is not curative and the median progression-free survival (PFS) in responders is still limited (4,5). To improve the response rate of anti-CD20 Ab therapy we developed a bispecific Ab (BsAb) named BIS20x3. BIS20x3 combines the antigen-specificity for CD20 on B-cells with the antigen-specificity for CD3ε on T-cells, within one Ab molecule (6). In contrast to rituximab, this BsAb can use the patients T-cell compartment for its anti-tumor effect by activating and retargeting T-cells to CD20-positive B-cells, independent of the TcR-defined specificity of T-cells.

Various BsAbs have been described over the last decade and *in vivo* anti-tumor activity has been shown in some cases in experimental clinical treatment settings (7, 8). One significant advantage of BIS20x3 over other BsAbs is that it targets B-cells, which are intrinsically capable of relaying costimulatory signals to T-cells because of their capacity as antigen presenting cells.

We have previously demonstrated that BIS20x3 induces efficient T-cell activation and allows retargeting of T-cells to CD20-positive B-cells, resulting in efficient killing of CD20-positive B-cells. We showed that BIS20x3-mediated crosslinking of CD20 at the cell surface of B-cells induces significant apoptosis in the B-cell lymphoma cell-line Ramos and we investigated the mechanism of T-cell activation mediated by BIS20x3 and B-cells (6,9). T-cell activation, via BIS20x3 targeted B-cells, involves integrin mediated costimulation of the T-cells and requires a functional NF-κB pathway in B-cells. We showed that B-cell mediated costimulation of T-cells is specifically related to targeting against CD20 and is less efficient for a CD19

In the present study, we proceeded to the *in vivo* application of BIS20x3. We used a well established NOD/scid mice animal model, developed to assess efficacy of eradication of human acute lymphoblastic leukemia (ALL) by human cytotoxic T lymphocytes. Immunodeficient NOD/scid mice are representative hosts for human

malignant and normal hematopoetic cells (10) and have also proven to be suitable for the study of human effector cells, like CTLs (11,12). As a model of human leukemia, the NOD/scid mice were engrafted with primary CD20-positive human leukemic cells from patients with precursor B-ALL as previously described (13).

Adults with ALL have a poor prognosis, demonstrated by a limited overall leukemia-free survival of 40-50% (14). B-lineage ALL blasts usually are CD20-negative. However, CD20-positive ALL can occur in a limited number of cases. This relatively refractory type of B-cell malignancy might be a possible target for BIS20x3 mediated CTL retargeting. Infusion of donor T-cells (donor lymphocyte infusion or DLI) is relatively successful in patients with relapsed chronic myeloid leukemia (CML), but is inefficient in ALL, probably due to limitation of the proliferative capacity of ALL-reactive T-cells (15). BsAb BIS20x3 in combination with CD20-positive ALL was used in this model to increase the cytolytic effectiveness of T-cells. Using this model, we observed effective T-cell retargeting, activation, and costimulation by BIS20x3, adding to the therapeutic potential of DLI therapy.

Materials and Methods

Patient and donor material.

After informed consent, leukemic cells were obtained by leukopheresis from a patient with ALL, characterized as B-lymphoid blast crisis from chronic myeloid leukemia (CML) cells expressing CD10, CD19 and CD20 and were named ALL-CM. ALL-CM cells were cultured as previously described (16). Peripheral blood was collected after informed consent from an HLA-identical unrelated healthy donor and PBMC fractions were isolated using Lymphoprep (Axis-Shield PoC AS, Oslo, Norway). Leukemic cells and donor PBMCs were cryopreserved in 10% dimethyl sulphoxide in liquid nitrogen. Approval was obtained from the Leiden University Medical Center review board for these studies. Non-CD20 specific CMV-CTLs, directed against the CMV matrix phosphoprotein 65 (pp65) were obtained as described before (17). Freshly isolated, unstimulated T-cells were isolated from the PBMC fractions obtained from peripheral blood from healthy individuals by MACS using antibody-coated magnetic beads (Dynal Biotech, Invitrogen, Breda, The Netherlands).

Cell lines.

The CD20-negative control cell lines Jurkat, AML 193 and U937, obtained from the ATCC (Rockville, MD, USA) and the T-cell line Jurkat AM/T (Dr. P. Schrier, University Medical Center Leiden, Leiden, The Netherlands) were cultured in Roswell Park Memorial Institute Medium (RPMI) 1640 culture medium (containing 25 mM Hepes and L-glutamin), supplemented with 14% heat-inactivated fetal calf serum (FCS) from Bodinco BV (Alkmaar, The Netherlands), 1 mM sodium pyruvate, 2 mM L-glutamin, 0.5 mM β-mercaptoethanol and 0.1 mg/ml gentamycin sulphate obtained from Bio-Whittaker (Verviers, Belgium) and 0.02 μg/ml fungizone (Bristol-Meyers, Woerden, The Netherlands). All cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Culture medium of the Jurkat AM/T cell line was supplemented with 0.5 mg/ml hygromycin B (Invitrogen).

Antibodies and reagents.

BIS20x3 and the parental anti-CD20 Ab B-ly1 was developed and characterized at the laboratory for Tumor Immunology, (Groningen, the Netherlands) and produced and purified by IQ Products (Groningen, The Netherlands) as described previously (6,9,18). The anti-CD3ε Ab 37-6673 (19) was a kind gift from Dr. R.A. van Lier (Academic Medical Centre, Amsterdam, The Netherlands). The mock-targeting BsAb BIS-1 and its $F(ab')_2$ fragment was developed in at the laboratory for Tumor Immunology, (Groningen, the Netherlands) as described before (7). Rituximab was

kindly provided by Roche Nederland BV (Woerden, The Netherlands). Commercial FITC- or PE-conjugated Abs (CD25, rabbit-anti-human (RAH) and goat-anti-mouse (GAM) used for FACS analysis were obtained from DAKO (Glostrup, Denmark). Recombinant human IL-2 was obtained from EuroCetus (Amsterdam, The Netherlands). CFSE was purchased from Sigma-Aldrich Chemie B.V (Zwijndrecht, The Netherlands). $Na_2{}^{51}CrO_4$ was purchased from GE Healthcare Europe GmbH (Munich, Germany).

BIS20x3 binding to ALL-CM cells.
ALL-CM cells were characterized by flow cytometry for CD20 expression. BIS20x3 binding was analyzed and compared to the parental B-ly1 anti-CD20 Ab. CD20 targeting of BIS20x3 was blocked by adding rituximab to the samples in various concentrations. BIS20x3 or B-ly1 binding was detected by means of secondary GAM-PE conjugated Abs, whereas rituximab was detected by secondary RAH-FITC conjugated Abs.

Analysis of T-cell activation.
T-cell activation was determined using the Jurkat T-cell subline Jurkat AM/T as described previously (6,20). Briefly, $1.0x10^6$ Jurkat AM/T cells were activated in 1.0 ml medium at 37°C, using the stimuli described, in the presence or absence of $0.2x10^6$ ALL-CM cells. T-cell activation was analyzed at 24 hours by detection of luciferase activity using the Luciferase Assay System (Promega, Leiden, The Netherlands) according to the instructions of the manufacturer. Luminescence was measured on the Anthos Lucy Microplate Luminometer and Photometer (Labtech International, Ringmer, UK). Experiments were performed in triplicate and luciferase levels were corrected for levels obtained in medium control samples.

Cytotoxicity assay.
Target cells were harvested, labeled with 50 µCi $Na_2{}^{51}CrO_4$ for 60 min at 37°C, washed twice, and added to various numbers of effector T cells. ALL-CM, Jurkat, AML 193 and U937 cells were used as target cells. The mixture of ^{51}Cr-labeled target cells and effector cells was subsequently treated with different concentrations of BsAb in presence or absence of blocking Abs in a final volume of 200 µl culture medium with 100 IU/ml IL-2 in 96-well U-bottomed microtiter plates. After 1-4 hr of incubation of target and effector cells at 37°C and 5% CO_2, 25 µl supernatant was harvested and measured in a luminescence counter (Topcount-NXT; Packard Instrument Co.). The mean percentage of specific lysis of triplicate wells was calculated as follows: specific lysis = [(experimental release – spontaneous release)/(maximal release – spontaneous release)] x 100.

ALL-CM proliferation and T-cell activation monitored in time.

Freshly isolated, unstimulated T-cells were purified from PBMCs from healthy donors and added to CFSE (5 μM) labeled target ALL-CM cells in various effector/target (E/T) ratios in the presence of 100 IU/ml IL-2. BIS20x3 was added where indicated at a concentration of 0.5 μg/ml. All samples were incubated for a maximum of 168 hrs (7 days) and test aliquots were taken for flow cytometric analysis at the indicated time points. Controls samples were also incubated containing ALL-CM alone, ALL-CM+BIS20x3, T-cells alone, T-cells+IL-2 and T-cells+IL-2+BIS20x3. For analysis of leukemia proliferation progression, the number of ALL-CM cells was calculated in each sample at the indicated time points. To calculate leukemic cell numbers, a constant amount of beads was added to each test sample. During flow cytometric analysis the analysis of a constant number of 500 beads was used to calculate the corresponding number of leukemic cells present. T-cell expansion was similarly analyzed, by counting the numbers of T-cell at the indicated time points, correcting for the number of beads and for the initial amount of effector cells. Mean CD25 expression per T-cell was determined by detecting CD25-PE MFI and correcting this result for background levels.

In vivo kinetcs of BIS20x3 binding.

A standard curve was created correlating the concentration of cell-bound BIS20x3 to the MFI of its secondary detecting Ab (goat-anti-mouse-PE). 25,000 cells/sample were incubated with a constant volume of BIS20x3 and GAM-PE, using increasing concentrations of BIS20x3. The logarithm for BIS20x3 concentration was correlated to the logarithm for MFI of GAM-PE. Using this standard curve, BIS20x3 concentrations in blood samples from injected NOD/scid mice were calculated. Mice were injected intravenously with different concentrations of BIS20x3 in 500 μl total volume and blood concentration kinetics of BIS20x3 was monitored in time by flow cytometric analysis of peripheral blood samples of individual animals.

NOD/scid mouse leukemia model.

Female NOD/scid mice aged 5 to 6 weeks were obtained from Bomholtgaard Breeding and Research Center (Ry, Denmark). As described previously, $1.0x10^7$ leukemic cells were injected into a lateral tail vein. Engraftment and progression of leukemia was monitored weekly by flow cytometric analysis of peripheral blood samples of individual animals (13). DLI was performed when between 0.5 and 5.0% leukemic cells were observed in peripheral blood of the animals. Before infusion, donor PBMC were thawed and washed twice in RPMI supplemented with 10% FCS. Viable PBMC (10^7) were injected intraperitoneally in 200 μl of RPMI/FCS. Where indicated, 50 μg of BIS20x3 was simultaneously injected, intravenously. When

required for analysis, or when animals were moribund, the mice were sacrificed by CO_2 inhalation.

Monitoring of leukemic cells and T-cells by flow cytometric analysis.
For analysis of peripheral blood of the animals, the total nucleated cell count (NCC) was determined in each blood sample using an automated Sysmex F820 blood cell counter (Sysmex Corporation, Kobe, Japan). Erythrocyte lysis was performed using NH_4Cl, and the samples were split. All aliquots were stained using phycoerythrin (PE)-conjugated anti-murine CD45 (Ly5; PharMingen, San Diego, CA, USA), and counterstained with either fluorescein isothiocyanate (FITC)-conjugated isotype control, anti-human CD10 or CD3, respectively (all Becton Dickinson (BD), San Jose, CA, USA). Analysis was performed on a FACscan cytometer (BD). Nucleated cells were identified based on their forward and sideward light-scatter properties. Absolute leukemic or T-cell counts (AC) were calculated as AC = NCC x the percentage of cells expressing the relevant membrane marker. T cells were gated on the basis of CD3 staining.

Results

BIS20x3 binding to ALL-CM.

BIS20x3 binding to the target cells ALL-CM was analyzed by flow cytometry and compared to binding of the parental Ab B-ly1 at an equal concentration. Detection of both Abs was performed by means of secondary GAM-PE Abs. Both BIS20x3 and B-ly1 Abs were added to the cells at a saturating concentration; binding to the ALL-CM cells appeared similar for both Abs (Fig. 1A). GAM-PE Ab alone did not induce significant background binding to ALL-CM cells. As depicted in Fig 1B, BIS20x3 binding to ALL-CM cells could be blocked by adding 1.0 µg/ml rituximab to the cells. Binding of BIS20x3 to ALL-CM cells therefore appeared to be target (CD20) dependent as it could be inhibited by target competing Abs. The optimal concentration for rituximab to inhibit BIS20x3 or B-ly1 binding was determined by analyzing BIS20x3 or B-ly1 binding in the presence of increasing amounts of rituximab. A minimum concentration of 1.0 µg/ml rituximab was necessary to completely decrease GAM-PE MFI to background levels. Apparently, 1.0 µg/ml rituximab was sufficient to block binding of 0.5 µg/ml BIS20x3.

T-cell activation and cytotoxicity.

In the NFAT-luciferase reporter T-cell line Jurkat AM/T, T-cell activation could be quantitatively determined by measuring luciferase activity. Fig 2A shows an increase in T-cell activation upon binding of BIS20x3 to CD3ε on the surface of the Jurkat AMT/cells in the presence of ALL-CM cells. Neither single BIS20x3, nor ALL-CM cells alone or control CD3 IgG Abs could induce this level of T-cell activation. To determine the T-cell retargeting capacity of BIS20x3, non-CD20 (CMV-pp65) specific CTLs were incubated together with ALL-CM cells in different E/T ratios in the presence of increasing concentrations of BIS20x3. For all three E/T ratios, the maximal amount of cytotoxicity was determined at a concentration BIS20x3 of 0.5 µg/ml (Fig 2B). Cytotoxicity increased in time for all three E/T ratios, up to 70% lysis at 3.5 hrs of co-incubation using 0.5 µg/ml BIS20x3 and an E/T ratio of 5:1 (Fig 2C). Importantly, no target cell lysis was induced in the absence of BIS20x3. Lysis could only be partially blocked by increasing amounts of either rituximab or B-ly1.

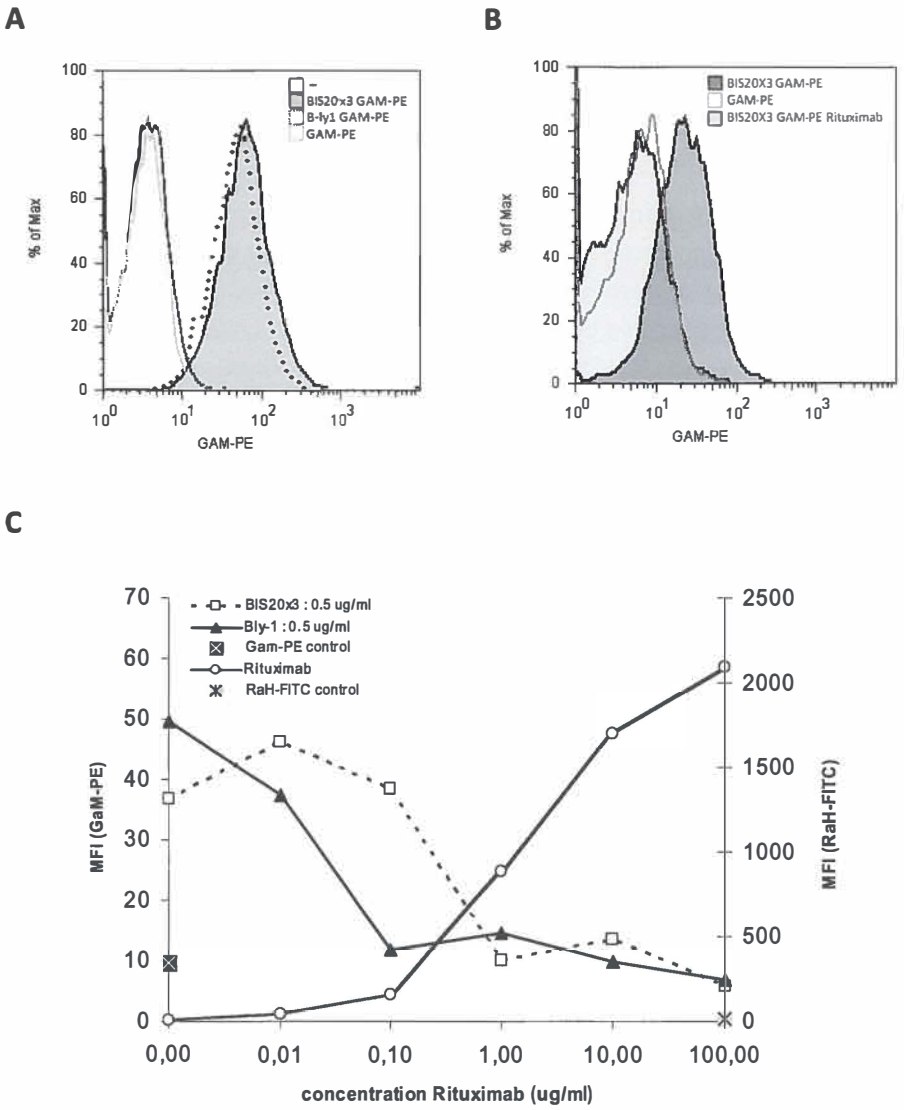


Figure 1. BIS20x3 binds efficiently and specifically to CD20 on ALL-CM cells. (A) ALL-CM cells were harvested, incubated for 30 min with 0.5 µg/ml BIS20x3 and compared to binding of the parental B-ly1 anti-CD20 Ab at equal concentrations. GAM-PE was also applied separately to exclude aspecific binding. (B) CD20 targeting of BIS20x3 was blocked by adding rituximab (1.0 µg/ml) to the samples. (C) Increasing concentrations of rituximab were used to compete for CD20 binding by either BIS20x3 or B-ly1 (0.5 µg/ml). Both GAM-PE and RAH-FITC secondary Abs were applied separately as controls for background fluorescence. Note the differences in mean fluorescent intensities (MFI) between both y-axes.

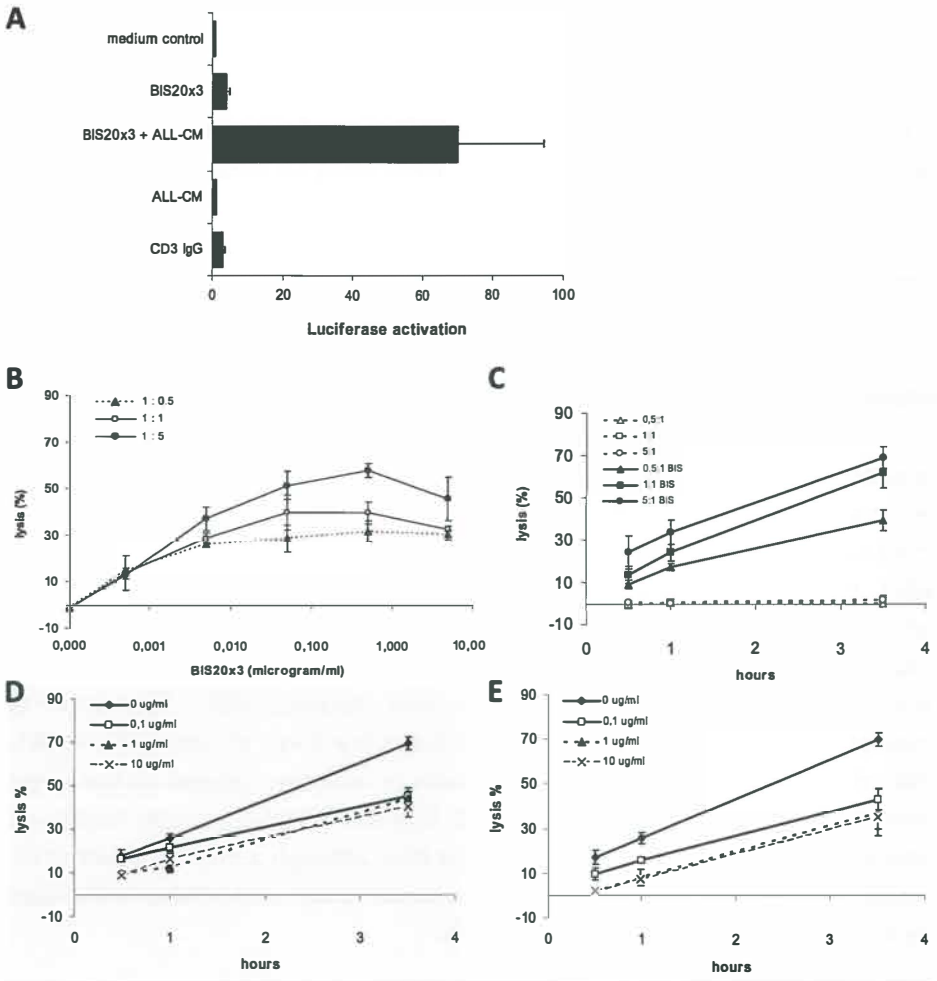


Figure 2. T-cell activation and the induction of cytytoxicity in ALL-CM cells. (A) Jurkat-AM/T cells, containing the NFAT-luciferase reporter gene, were cultured with 0.5 µg/ml BIS20x3 alone or in the presence of ALL-CM cells, at a T-cell/ALL ratio of 5:1. As a control Jurkat-AM/T cells were also cultured with ALL-CM cells alone. As a positive control, T-cells were stimulated with 5 µg/ml Ab 37-6673 (anti-CD3ε). Luciferase activity of the medium control was set at 1.0 arbitrary unit and each condition was corrected for (divided by) the medium control accordingly. The mean percentage of triplicate assays (+ SEM) is displayed for each condition. (B) ALL-CM cells were harvested, labeled with 50 µCi $Na_2{}^{51}CrO_4$, and added to effector CTLs in E/T ratios of 0.5:1, 1:1 or 5:1. The mixture of ^{51}Cr-labeled target cells and effector cells was treated with increasing concentrations of BsAb. After two hours of incubation at 37°C, supernatant was harvested and the percentage of lysis was determined according to the described calculation method. The mean result of quadruplicate assays is displayed for each ratio and concentration along with the SEM. (C) ALL-CM cells were treated as described using different E/T ratios and in the presence of 0.5 µg/ml BIS20x3 where indicated. The percentage of lysis was determined at various time points. (D) ALL-CM cells were treated with 0.5 µg/ml BIS20x3 at an E/T ratio of 1:1 as described, with the addition of different concentrations of blocking rituximab Ab. The percentage of lysis was determined at various time points. (E) CD20 blocking was also performed using B-ly1 in stead of rituximab as competing Ab. Again, the mean result of quadruplicate assays is displayed for each ratio, concentration or time point along with the SEM.

CD20 specific targeting.

Because lysis by BIS20x3 retargeted CTLs could not be completely inhibited by competing CD20-binding Abs in cytotoxicity experiments, we further explored CD20-specificity of BIS20x3. In Fig 3A-D, four different cell types are shown, the first being the CD20-positive ALL-CM, the other three being CD20-negative control cell lines (T-cell leukemia cell line Jurkat; acute myeloid leukemia cell line AML 193; monocyte cell line U937). All four cell lines were incubated with non-CD20 specific CTLs at three different E/T ratios in the presence or absence of 0.5 µg/ml BIS20x3 for 4 hours. In none of the CD20-negative cell lines, lysis was achieved as compared to lysis levels induced in the ALL-CM cells. Only in the Jurkat T-cell line low levels of cytotoxicity were seen, possibly due to direct anti-CD3 induced apoptosis (21,22).

In addition, to exclude that the BIS20x3 mediated anti-leukemic cytotoxicity did not occur as a result of interaction with Fc-receptors expressed by the target cells, a mock-targeting BsAb, named BIS-1, and its $F(ab')_2$ fragment derivate were used. BIS-1 targets the pancarcinoma/epithelium associated glycoprotein EGP-2, a protein not present on ALL-CM cells (7). ALL-CM cells were incubated with non-CD20 specific CTLs in the presence or absence of 0.5 µg/ml BsAb. In the presence of BIS20x3, high levels of target cell lysis were obtained within four hours of incubation. Treatment with BIS-1 IgG resulted in low levels of cytotoxicity in ALL-CM cells, most likely due to interaction with Fc-receptors present on the target cells. Samples incubated with the F(ab')2 fragment of BIS-1 showed hardly any residual cytotoxicity (Fig 3E). We concluded that, although a minimal effect of Fc-receptor binding cannot be excluded, the cytotoxic effect on ALL-CM cells mediated by BIS20x3 was predominantly CD20 specific.

BIS20x3 mediated inhibition of ALL-CM proliferation using unstimulated T-cells.

To study T-cell activation, expression of the T-cell activation marker CD25 was analyzed. CD25 expression increased transiently with maximal expression after 48 hours of incubation after which expression levels declined. Interestingly, CD25 expression was highest in samples with the lowest E/T ratio. In samples treated with BIS20x3 only, CD25 expression on T-cells increased comparable to background levels on unstimulated T-cells (Fig 4A). To further study T-cell activation and ALL-CM and T-cell proliferation, freshly isolated, unstimulated T-cells were added to CFSE labeled target ALL-CM cells in various E/T ratios in the presence or absence of BIS20x3. T-cells expanded in all samples but the sample with T-cells alone, probably due to the presence of IL-2. However, expansion of T-cells was significantly higher in samples containing BIS20x3. The difference in E/T ratio was not of influence for the outcome of expansion.

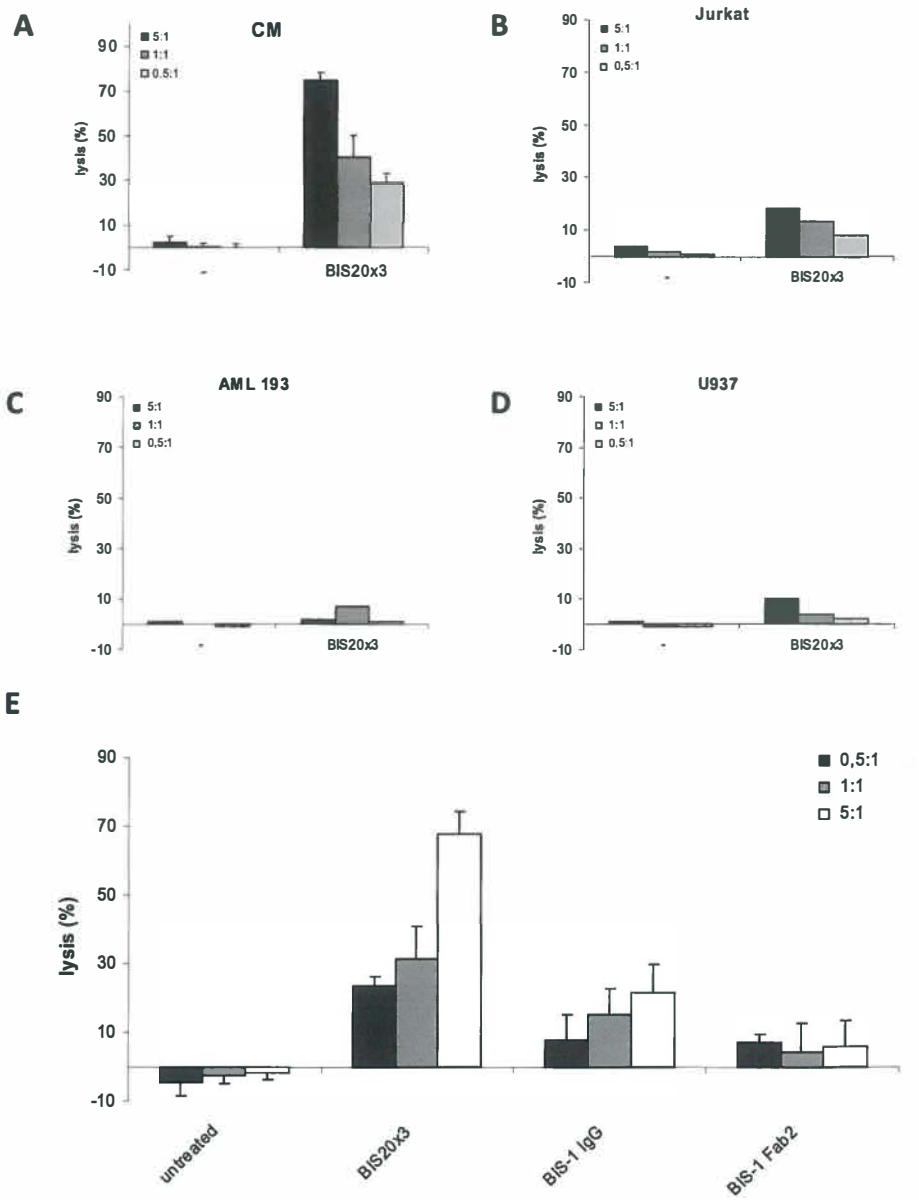


Figure 3. BIS20x3 effects are CD20 specific. (A-D) ALL-CM, Jurkat, AML 193 or U937 cells were harvested, labeled with 50 µCi $Na_2{}^{51}CrO_4$, and added to effector CTLs at E/T ratios of 0.5:1, 1:1 or 5:1. Samples were treated where indicated with 0.5 µg/ml BsAb. After 4 hrs of incubation at 37°C, supernatant was harvested and the percentage of lysis was determined according to the described calculation method. (E) ALL-CM cells were treated as described with 0.5 µg/ml of the indicated BsAb at different E/T ratios. The mean result of quadruplicate assays is displayed for each ratio and concentration along with the SEM.

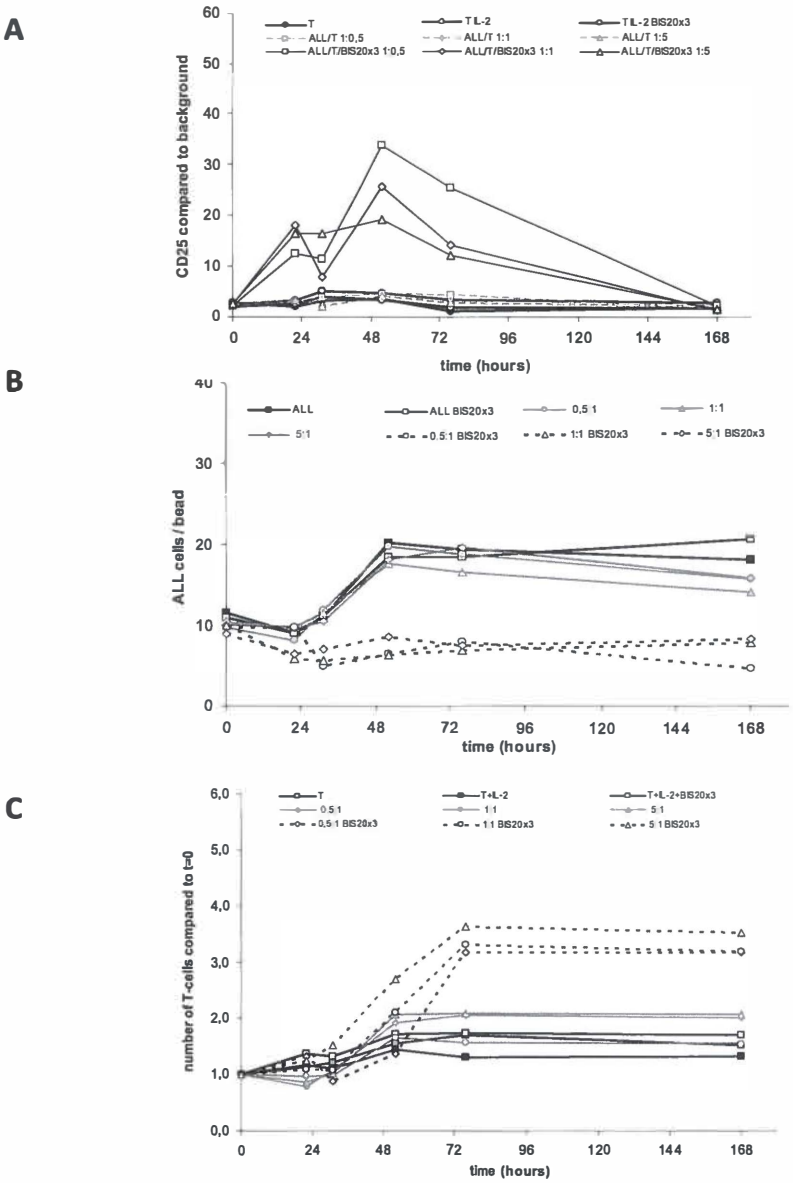


Figure 4. BIS20x3 mediates T-cell activation and expansion and induces inhibition of ALL-CM proliferation. Freshly isolated, unstimulated T-cells were purified from PBMCs from healthy donors and added to CFSE (5 µM) labeled target ALL-CM cells in E/T ratios of 0.5:1, 1:1 or 5:1. BIS20x3 was added where indicated at a concentration of 0.5 µg/ml. All cells were incubated for a maximum of 168 hours (7 days) and test aliquots were taken for flow cytometric analysis at the indicated timepoints. As controls were incubated; ALL-CM alone, ALL-CM+BIS20x3, T-cells alone, T-cells+IL-2 and T-cells+IL-2+BIS20x3. (A) Mean CD25 expression per cell was determined on CFSE negative cells (T-cells) by staining with CD25-PE and correcting the MFI for background levels. (B) T-cell expansion was analyzed at the indicated time points by counting the numbers of CFSE negative cells, correcting for the counted number of beads and additionally correcting for the starting ratio number of effector cells. For analysis of leukemia proliferation progression, CFSE positive ALL-CM cells were counted in each sample and also corrected for the counted number of beads.

Maximal expansion was observed after 72 hours of culturing. Leukemia cell proliferation was similarly analyzed. In the presence of BIS20x3 no progression was seen in ALL-CM proliferation. In contrast, samples not containing BIS20x3 showed ALL-CM cells expanding up to a doubling of the number of lymphoma cells (Fig 4B). ALL-CM cell proliferation inhibition seemed to be independent of the E/T ratio. After 48 hours no proliferation was seen in any of the samples.

The kinetics of BIS20x3 binding in vivo.

A standard curve was created correlating the concentration of cell-bound BIS20x3 to the MFI of its secondary detecting Ab (goat-anti-mouse-PE) (Fig. 5A). The logarithm for BIS20x3 concentration directly correlated to the logarithm for MFI of goat-anti-mouse PE (Fig. 5B). Using this standard curve, BIS20x3 concentrations in blood samples from injected NOD/scid mice were calculated. Four mice were injected intravenously with 0, 50, 100 and 200 µg BIS20x3 and at the indicated time points the blood concentration of BIS20x3 within the mice was determined. Concentrations were the highest at eight hours after infusion; 96 hours after infusion no BIS20x3 could be detected anymore for all concentrations administered (Fig 5C).

In vivo inhibition of leukemia proliferation by BIS20x3

To study the *in vivo* therapeutic effects of BIS20x3 in an animal model, ALL-CM leukemic cells were inoculated in NOD/scid mice at day 0 and progression of leukemia was monitored for almost 12 weeks (82 days). DLI was performed after 45 days when sufficient amounts of leukemic cells were observed in peripheral blood of all animals. Four mice were treated with DLI only and four mice were additionally treated with 50 µg of BIS20x3. One animal in the BIS20x3 group was sacrificed shortly after treatment with DLI and BIS20x3 because of development of additional lymphoma. For analysis of peripheral blood of the animals, the total nucleated cell count (NCC), and the absolute leukemic cell counts (AC) were determined in each blood sample as described. Fig 6A shows the progression of leukemia in four mice treated with DLI alone. In all mice continuous progression of leukemic cells was seen after infusion, although differences in the extent of progression were observed. In Fig 6B, the effect of addition of BIS20x3 can be seen, showing a prolonged delay of progression of leukemia up to 65 days after inoculation. Fig 6C shows the mean ACs for all mice treated with DLI with or without BIS20x3. Both leukemic cell counts and progression free survival were significantly lower in the group treated with BIS20x3 suggesting a beneficial therapeutic effect of BIS20x3. The ACs of T-cells were also calculated for both settings but were relatively low in the DLI only group and not detectable in the DLI plus BIS20x3 group (results not shown).

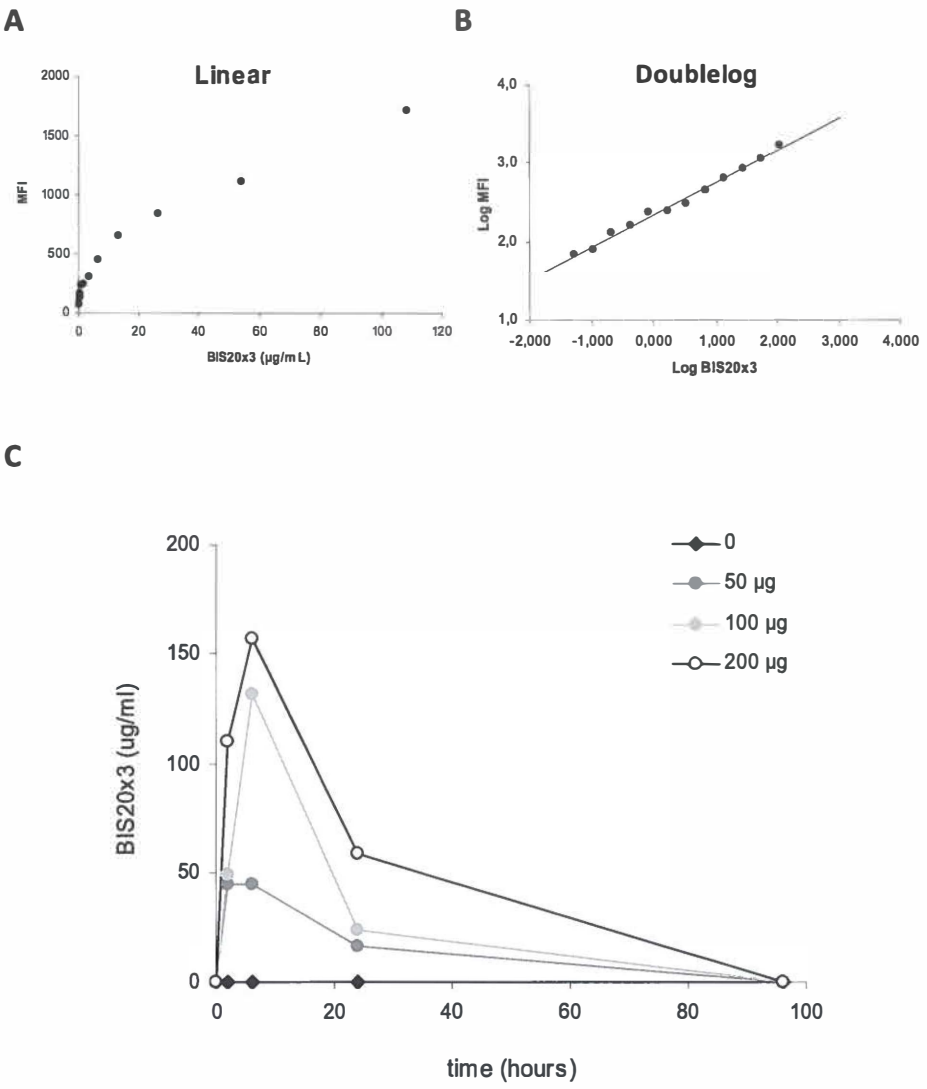


Figure 5. The kinetics of BIS20x3 in NOD/scid mice. (A) 25.000 cells were incubated with a constant volume of BIS20x3 and GAM-PE, using increasing concentrations of BIS20x3 in the presence of 10 µg/ml mouse serum. A standard curve was created correlating the concentration of cell-bound BIS20x3 to the increasing MFI of GAM-PE. (B) The logarithm for BIS20x3 concentration was correlated to the logarithm for MFI of GAM-PE. (C) Using the standard curve and the corresponding equation, BIS20x3 concentrations in blood samples from injected NOD/scid mice were calculated. Mice were injected intravenously with different concentrations of BIS20x3 in 500 µl of total volume and blood concentration kinetics of BIS20x3 was monitored in time by determining MFI of GAM-PE bound to BIS20x3 detected in peripheral blood of individual animals.

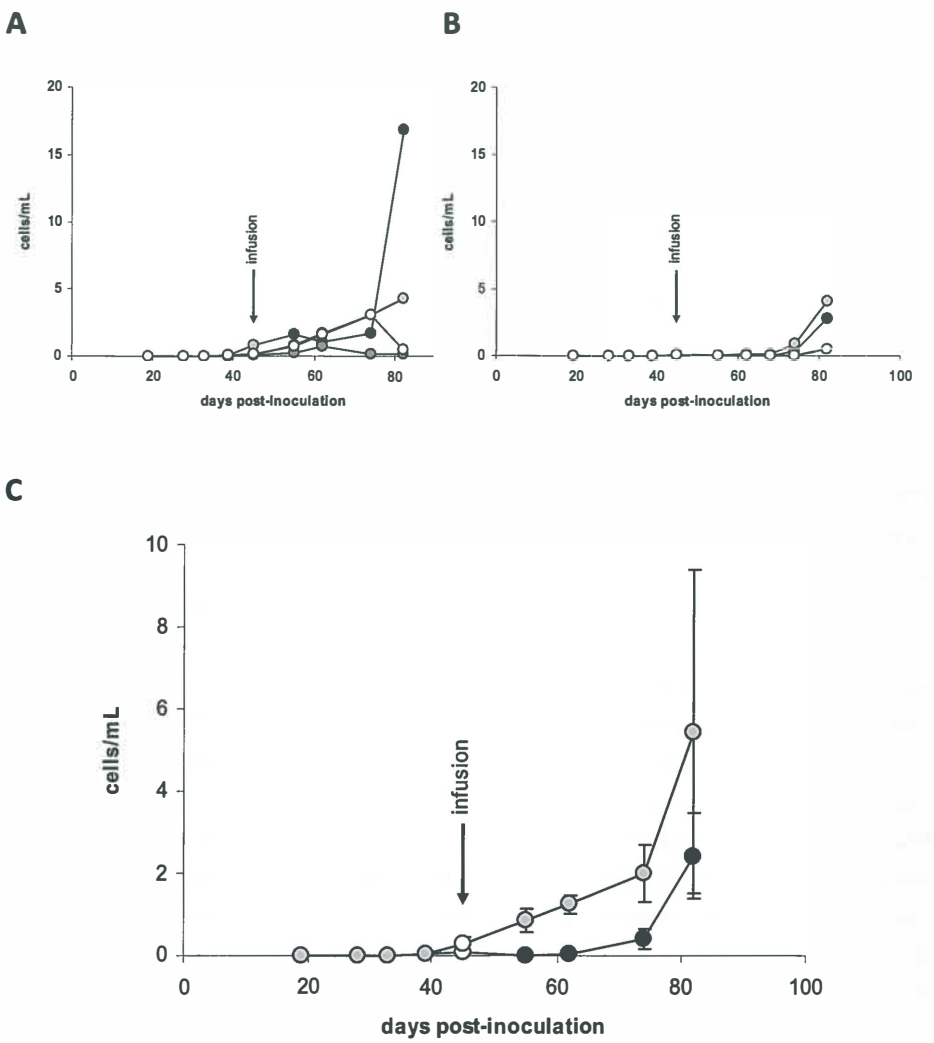


Figure 6. In vivo inhibition of leukemia proliferation. $1.0x10^7$ leukemic ALL-CM cells were injected into a lateral tail vein of female NOD/scid mice. Engraftment and progression of leukemia was monitored weekly by flow cytometric analysis. At day 45, $1.0x10^7$ viable PBMC were injected intraperitoneally in 200 µl of RPMI/FCS. Where indicated, 50 µg of BIS20x3 was simultaneously injected, intravenously. The total NCC was determined in each blood sample based on their forward and sideward light-scattering properties. Subsequently, all aliquots were stained using PE-conjugated anti-murine CD45 and counterstained with either FITC-conjugated isotype control, anti-human CD10. Absolute leukemic cell counts were calculated as described in the materials and methods section. (A) Progression of leukemia was determined in four animals (◎ ○ ◎ ●) treated with DLI alone. (B) Progression of leukemia was also determined in three animals (○ ◎ ●) treated with DLI in combination with 50 µg of BIS20x3 per animal. (C) Mean counts of leukemic cells were determined for mice treated with DLI alone (◎) and mice treated with DLI plus BIS20x3 (●) and results were depicted along with the SD.

Discussion

In this study, we demonstrated the *in vitro* and *in vivo* capacity of BIS20x3 to redirect T-cells towards patient-derived CD20-positive leukemia cells. We showed that BIS20x3 can activate and retarget freshly isolated T-cells to patient-derived leukemia cells in an efficient, rapid and highly lytic manner. Upon BIS20x3 binding the isolated T-cell population expanded and suppressed leukemia cell proliferation *in vitro*. Finally, *in vivo* data showed that BIS20x3 treatment, in combination with DLI, resulted in significant lower leukemic cell counts and progression free survival.

BIS20x3 was principally designed to create an entity not dependent on the patients humoral immune response, but which derives its anti-tumour effect from the cytocidal activity of retargeted T-cells in a CD20-restricted manner. Previously, we have demonstrated that cell-cell interactions between B-cells and T-cells, mediated through binding of BIS20x3 and closely resembling T-cell costimulatory events, resulted in a highly efficient activation of T-cells into CTLs (9). Furthermore, CD20 is described to be physically and functionally coupled to MHC class II molecules and we have demonstrated recently that CD20 and HLA-DR signaling pathways are intertwining (23,24). Membrane rearrangement and the formation of lipid rafts are essential for MHC class II signaling and formation of the immune synapse. We believe that CD20 is physically implicated in this process. CD20 may support immune synapse formation, thereby facilitating essential transmembrane signaling needed for efficient B-cell costimulation via HLA-DR/T-cell receptor interaction. Considering this, we postulate CD20 to be an optimal target for antibody mediated therapies, especially involving T-cell activation and B-cell costimulation. BIS20x3 might induce additional effects in T-cells and (tumour)B-cells *in vivo*, as compared to conventional Abs like rituximab, thereby enhancing its therapeutic options.

In the present study, we wanted to explore the possibilities of BIS20x3 in an *in vivo* setting by using a known model of ALL engraftment in NOD/scid mice (13). To ensure that BIS20x3 effects were antibody specific in this model, a series of *in vitro* experiments were performed before proceeding to an *in vivo* setting. In short; BIS20x3 was shown to bind to ALL-CM cells as efficiently as its parental Ab B-ly1 and this binding was CD20 specific, since it could be blocked by the CD20 competitive Ab rituximab. T-cell activation appeared to be efficient and BIS20x3 could mediate efficient CTL retargeting and rapid lysis. Interestingly, although non-CD20 specific CTLs have cytolytic potential, they did not induce substantial lysis in the non-targeted setting without BIS20x3 present. These results showed that antibody specific T-cell retargeting and ALL-CM lysis can be achieved by means of

BIS20x3 treatment. Interestingly, blocking of CD20 binding by addition of rituximab or B-ly1 inhibited ALL-CM lysis only partially, although Ab concentrations were used up to ten times higher than those needed to completely block binding. Probably, Ab binding is and on-off equilibrium, therefore BIS20x3 Abs are still capable of reaching their target and retargeting a sufficient number of CTLs to induce cell death. These findings suggest that even low availability of CD20 molecules and short contact time might be enough to induce efficient cytotoxicity via BIS20x3. CD20 specificity was ensured in our model, showing virtually no lysis in CD20-negative cell lines neither in the presence nor in the absence of BIS20x3. And although a minimal effect of Fc-receptor binding could not be excluded, the cytotoxic effect on ALL-CM cells mediated by BIS20x3 was predominantly CD20 specific.

Subsequently, we studied the potential and kinetics of BIS20x3 activating freshly isolated T-cells, using ALL-CM cells as target *in vitro*. CD25 expression increased up to 30 times on unstimulated T-cells upon co-culturing with BIS20x3 and ALL-CM. Increase in CD25 expression was highest using low E/T ratios, suggesting that increased cell-cell interactions, as can be expected with lower E/T ratios, may result in better costimulation and more efficient T-cell activation. E/T ratios appeared to have less effect on T-cell expansion. T-cell expansion stopped after 72 hours, suggesting lack of sufficient growth factors in the culture medium or a decrease of available BIS20x3. Interestingly, the number of effector cells was also not influencing leukemia cell proliferation. In all three samples containing ALL-CM cells, T-cells and BIS20x3, leukemia proliferation was inhibited equally. Proliferation in the other samples stopped at 48 hours of culturing, again suggesting exhaustion of the culture medium or achieving equilibrium in proliferation and spontaneous cell death.

We continued our study by determining the *in vivo* kinetics of BIS20x3 infused in NOD/scid mice. BIS20x3 concentrations were calculated in peripheral blood and appeared to be highest 8 hours after intravenous infusion. After three days, no BsAb was left to be detected, suggesting either degradation of the Ab or extravasation of Ab-bound cells out of the blood stream. These time periods correspond with the *in vitro* data of duration of T-cell expansion and activation, making BIS20x3 degradation more likely, since extravasation plays no role in the *in vitro* setting. These results suggest the necessity of repeated infusion of Ab when used for therapeutic options, where maintained levels of Ab are usually necessary.

To observe the therapeutic effects of BIS20x3 *in vivo*, eight NOD/scid mice were inoculated with ALL-CM cells. It was promising to find that ACs of leukemic cells

were lower and progression free survival was longer in the presence of the BsAb, suggesting a beneficial effect of BIS20x3. Apparently T-cell activation and BIS20x3 restricted anti-leukemia effects can be obtained in this *in vivo* model. Leukemia proliferation is inhibited significantly for up to 80 days. Possibly, repeated infusions of BIS20x3 might prolong this effect.

T-cells could not be detected in the BIS20x3 treated mice, suggesting extravasation of the T-cells into the organs or massive T-cell death due to extreme activation. Both effects could be harmful, resulting in organ failure or tumor lysis syndrome. Therefore advanced experiments using lower concentrations Ab or $F(ab')_2$ fragments of BIS20x3 are necessary. As seen in the *in vitro* experiments, higher amounts of BsAb are not always more efficient and titration experiments have to be performed.

All together, BIS20x3 is confirmed to be efficient in activating and retargeting T-cells, not only to B-cell lines but also to patient derived CD20-positive leukemic cells. Cytotoxicity levels achieved in these cells are high and the *in vivo* therapeutic efficacy of BIS20x3 seems promising.

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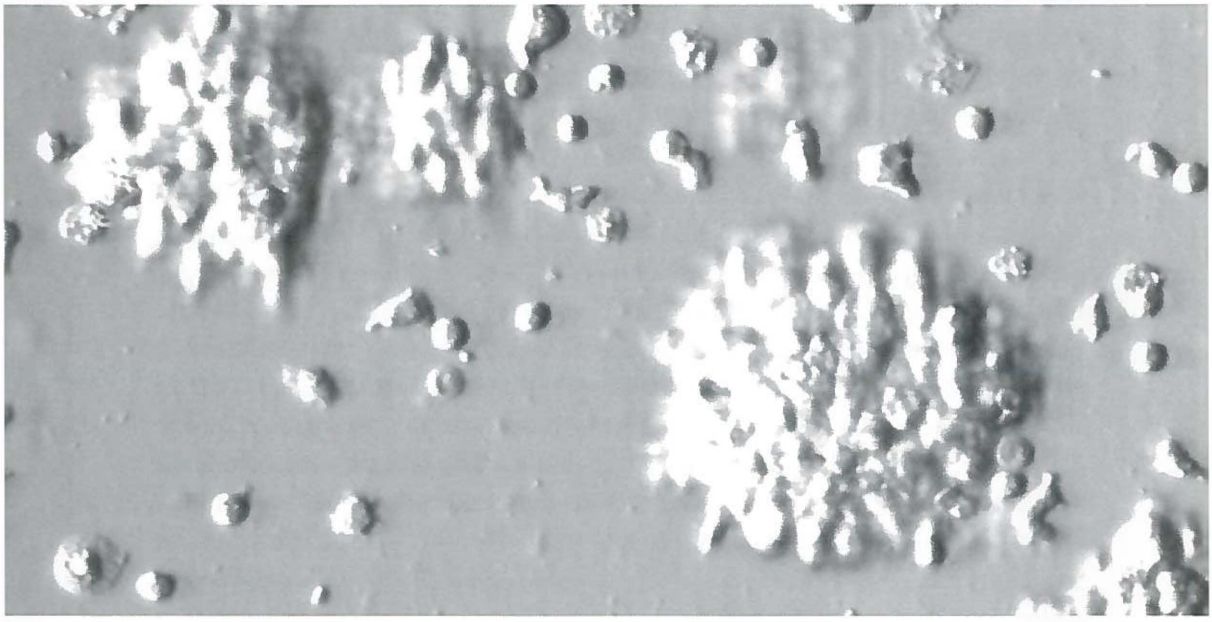
Chapter 7



Summary.

Summary of the thesis.

The application of rituximab has been shown to improve clinical outcome in patients with various types of B-cell mediated diseases. However, for many patients, rituximab treatment is not curative and the median progression-free survival in responders is limited. In an effort to improve the response rate of CD20 targeting Ab therapy, the BsAb BIS20x3 was developed, combining the antigen-specificity for CD20 on B-cells with the antigen-specificity for CD3ε on T-cells. In contrast to rituximab, this BsAb uses autologous T-cells within the patient for its anti-tumor effect. It acts by activating and retargeting T-cells to CD20 positive B-cells, independent of the TCR/MHC-specificity of the T-cells. In this thesis we provided evidence that choosing CD20 as a target for bispecific immunotherapy turned out to be a very promising one, since several CD20 related mechanisms contributed to an optimal activation of T-cells.

As an introduction to the thesis, in **chapter 1**, established knowledge and recent findings in the field of CD20 structure, function, possible ligands, possible complex molecules and various downstream signaling pathways of interest, have been put into order. Although there have been many speculations on the possible physiological function of CD20, its precise biological role still remains unknown. From the moment that CD20 was first described by Clark et al in 1985, CD20 is thought to play a role in B-cell development. The state of phosphorylation of CD20 was suggested to influence various intermolecular interactions, was associated with the regulation of tyrosine kinase activity, but also appeared to involve MAPK activity and NF-κB activity, regulating gene transcription in B-cells. The predicted structure of CD20 and its capacity to multimerize suggested that it may function as an ion channel. Crosslinking of CD20 regulated calcium conductance in B-cells. The regulation of calcium conductance by CD20 was shown to be dependent on the translocation of CD20 to lipid rafts. The movement of CD20 towards lipid rafts appeared to induce various effects. For example, the induction of apoptosis was largely dependent on the translocation of CD20 into rafts. Other established findings were that CD20 translocation into lipid rafts can influence the phosphorylation state of several tyrosine kinases, among which the Src-kinase family members Lyn, Fyn and Lck, Especially Lyn played a role in the capacity of CD20 to sensitize malignant B-cells to chemotherapy. Different types of anti-CD20 Abs (type I and II) displayed different capacities in lipid raft translocation, calcium conductance, complement activation and apoptosis. Considering the influence CD20 has on B-cell membrane composition and its close relation to the tetraspanin family and other MS4A family members we suggest that the physiological role of

CD20 may be found in organizing membrane composition. CD20 is known to physically interact with HLA-DR on B-cells and both induced common signaling pathways. We hypothesized that CD20 might play a role in efficient activation of the B-cell via the HLA-DR / antigen presentation pathway, being involved in signaling from the immunological synapse. Close interaction of CD20 with the MHC class II platform may activate a CD20 mediated cascade, resulting in signals involved in costimulation, cell survival and chemosensitization. This hypothesis supports our belief that CD20 might be an attractive target for BsAb immunotherapy.

In **chapter 2** the isolation of the T-cell retargeting Ab BIS20x3 from quadroma culture supernatant was described. The characterisation of both antigen binding moieties showed that BIS20x3 specifically bound to both CD3ε on T-cells and CD20 on B-cells and induced specific signaling via both target antigens. Furthermore, BIS20x3 was capable of inducing efficient activation and retargeting of cytotoxic T-cells towards CD20 expressing B-cell lines. The remarkable efficiency of BIS20x3 is thought to be due to costimulation of the T-cell, a result of the antigen presenting capacities of the B-cell. This phenomenon was further investigated in **chapter 3.** We demonstrated that BIS20x3-instigated T-cell / B-cell interaction caused a dose dependent activation of T-cells, many times stronger than T-cell activation induced by monovalent anti-CD3 Abs. Additionally, the interaction resulted in a significant upregulation of ICAM-1 on B-cells. T-cell activation was found to be dependent on the interaction of ICAM-1 with LFA-1 as well as on activation of the NF-κB pathway. The lytic potential of freshly isolated T-cells activated via BIS20x3 appeared also to be dependent on NF-κB signaling in the target B-cells. Costimulatory signaling effects were shown to be specifically related to CD20 targeting since targeting CD19, by a CD3xCD19 directed BsAb, was significantly less effective in inducing T-cell activation and T-cell mediated B-cell lysis. Apparently, specific signaling via CD20 resulted in both intracellular and membrane changes in B-cells responsible for their efficient lysis by effector T-cells.

Signaling via CD20 appeared also to be responsible for multimerization of other molecules within lipid raft domains as was shown for the death receptor Fas in **chapter 4**. In this chapter, evidence was provided that rituximab can sensitize lymphoma B-cells to Fas-induced apoptosis. Additionally, it was demonstrated for the first time that rituximab-induced apoptosis involved the death receptor pathway and proceeded in a caspase-8 dependent manner. A direct death receptor / ligand interaction appeared not to be involved in CD20-mediated cell death. Instead, we showed that rituximab-induced apoptosis involved spontaneous membrane clustering of Fas molecules leading to formation of the death inducing

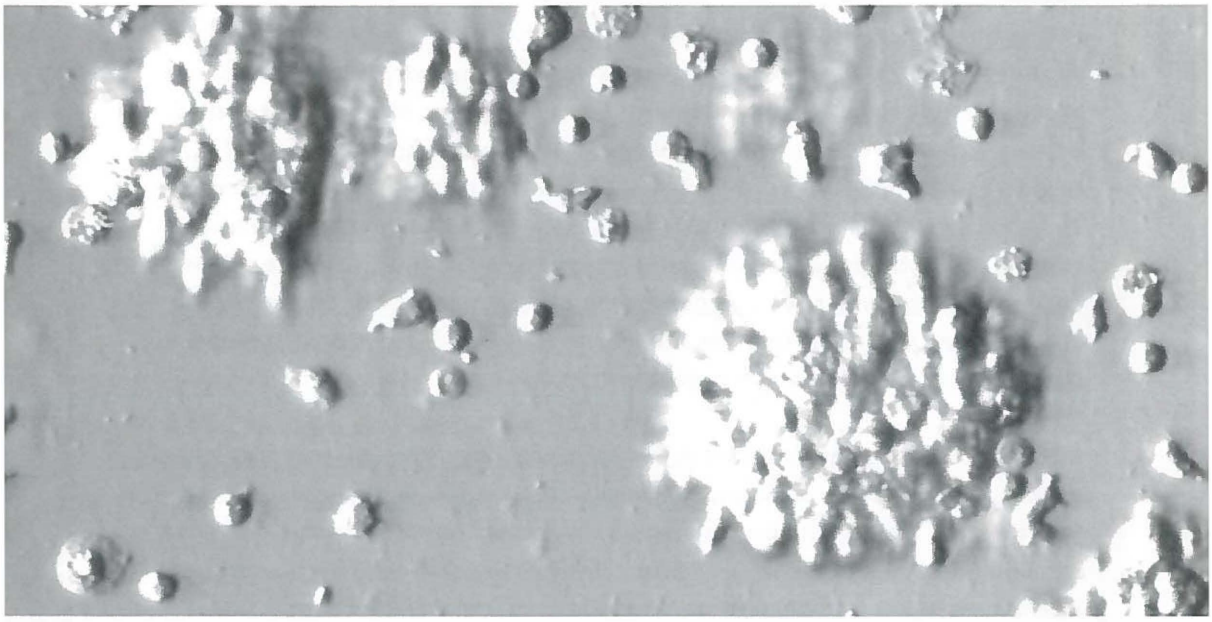
signaling complex (DISC) and downstream activation of the death receptor pathway. In this study we presented evidence for the involvement of the death receptor pathway in rituximab-induced apoptosis of Ramos B-cells with concomitant sensitization of these cells to Fas-mediated apoptosis via CD20-induced Fas multimerization and recruitment of caspase-8 and FADD to the DISC. It is very likely that CD20 is present in a multimolecular complex receiving and passing on signals from unknown related receptors without the need for direct association. In **chapter 5**, signaling capacities of both CD20 and HLA-DR were examined and compared. By assessment of apoptosis, calcium conductance, Fas sensitization, lipid raft formation and cytoskeleton rearrangements we showed that CD20 and HLA-DR share important cascades in B-cell activation in Ramos cells. In addition, functional interaction between CD20 and HLA-DR was indicated using the HLA-DR negative BLS-2 cell line, which was insensitive for Rituximab-induced apoptosis, despite abundant CD20 expression. Restoring HLA-DR expression re-introduced sensitivity to apoptosis induced via CD20. Furthermore, HLA-DR stimulation could induce CD20 raft translocation in Ramos cells, independent of CD20 triggering. Membrane rearrangement and the formation of lipid rafts appears essential for MHC class II signaling and formation of the immune synapse. We postulated that CD20 may have a biological function in amplifying the signals of related receptor molecules like HLA-DR. Maybe, like tetraspanins, CD20 can facilitate immune synapse formation and receptor signaling in B-cells.

Our hypothesis that BIS20x3 may be an ideal tool for T-cell retargeting therapy is strengthened by the three facts that 1) BIS20x3 can efficiently activate and retarget cytotoxic T-cells to malignant B-cells *in vitro*, without the need for additional costimulation, since this is provided by the B-cell itself 2) CD20 stimulation makes B-cell more sensitive to Fas induced apoptosis, one of the major cytotoxic effector mechanisms of activated T-cells and 3) CD20 shares significant homology with and even participates in HLA-DR signaling and perhaps immune synapse formation

We decided to further explore the possibilities of retargeting immunotherapy via BIS20x3 in an experimental *in vivo* setting. In **chapter 6** we demonstrated the capacity of BIS20x3 to activate T-cells towards patient-derived CD20 positive leukemia cells. In *vitro,* BIS20x3 specifically opsonized CD20 positive ALL cells and allowed efficient redirected cytotoxicity of CTLs. We showed that BIS20x3 induced efficient T-cell activation *in vitro*, as determined by CD25 expression. T-cell populations expanded and leukemia progression was strongly inhibited in cell culture studies. Subsequently, we introduced BIS20x3 into an *in vivo* CD20 positive leukemia NOD/SCID mouse model, developed to assess the anti-leukemic efficacy of donor lymphocyte infusion (DLI). In these experiments, BIS20x3 treatment, in

combination with DLI resulted in significant lower leukemic cell counts, compared to counts obtained with DLI treatment alone, and progression free survival was increased to almost 80 days. All together, BIS20x3 is confirmed to be efficient in activating and retargeting T-cells, not only to B-cell lines but also to patient derived $CD20^+$ leukemic cells. Cytotoxicity levels achieved in these cells are high and the *in vivo* therapeutic efficacy of BIS20x3 seems promising. BIS20x3 appears to be a promising clinical tool in B-cell depleting therapy via CD20 retargeting, since multiple signaling cascades aid to the activation of cytotoxic effector cells and the sensitivity of the target cells.

Chapter 8



General discussion.

General discussion

CD20 as a target for BsAb-mediated B-cell depletion.

In this thesis we describe functional aspects of a BsAb, BIS20x3, directed specifically against both CD3ε on T-cells and CD20 on B-cells (**chapter 2 and 3**). In general, binding of an anti-CD3 directed BsAb to the TCR/CD3 complex, leads to T-cell retargeting and cytotoxicity *in vitro*, irrespective of T-cell receptor specificity and MHC expression / antigen presentation. However, lack of costimulatory molecules on tumour cells results in insufficient activation of autologous T-cells in various (pre)clinical studies (1-3). To warrant *in vivo* effectiveness of this therapeutic modality, various attempts were made to meet the need for costimulation, e.g. by adding subcutaneous IL-2, injecting *in vitro* preactivated effector cells or combining treatment with CD28 costimulatory mAbs (2,4,5). B-cell depletion therapy was hypothesized to be specifically suited for T-cell retargeting BsAb therapy, since B-cells are natural APCs endowed with natural costimulatory capacity towards T-cells. Glennie *et al* investigated a panel of anti-CD3 BsAbs, targeting neoplastic B-cells (6). The efficiency by which these BsAb induced redirected cytotoxicity *in vitro* was consistently: anti-MHC class II > anti-CD19 > anti-IgM > anti-CD22, whereas *in vivo* only anti-IgM derivates were capable of prolonging survival of tumour-bearing animals. They suggested that the potency of these BsAbs specifically related to their signaling activity on target tumour cells.

CD20 as a target was not included in this panel of BsAbs, although we think CD20 is especially suitable for bispecific targeting. The specific features and signaling moieties of CD20 can trigger B-cells in becoming attractive targets for immunotherapy. For example, CD20 is highly expressed on B-cells and is not internalized upon Ab binding, as compared to other targets, as CD19 (7). Furthermore, CD20 signaling enhances B-cell sensitivity to other cell death inducing agents (8). In this thesis we showed that targeting CD20 can sensitize B-cells to Fas-mediated apoptosis and also induce direct CD20-mediated apoptosis (**chapter 4 and 5**). In addition, in our studies we provide evidence that BIS20x3 can use various membrane and intracellular capacities of CD20 to trigger the target B-cell in functioning as a professional APC. In **chapter 3** we showed that T-cell activation is efficient because CD20-activated B-cells can provide sufficient costimulatory signals to increase and maintain T-cell activation. This appeared to be dependent on specific CD20-mediated NF-κB signaling effects and T-cell activation levels could not be achieved by CD19xCD3 bispecific antibody treatment. It appeared that CD20 targeting is unique in its capacity to modify circumstances for T-cell activation and B-cell susceptibility.

CD4 and CD8 T-cell responses.

One of the early events in T-cell activation via BsAbs is the expression of CD25 (IL-2R), followed by release of IFN-γ, TNF-α, IL-4, IL-6 and IL-10 (9,10). These are interleukin patterns typical for both CD4 and CD8 T-cell activation cascades. Although CD3 binding results in activation of cytotoxic T-cells, this does not necessarily result in a CD8 restricted cytolysis of target cells. Buhler *et al* showed that both CD4- and CD8-positive T-cells were activated upon CD3-stimulation via a bispecific antibody (11). Tani *et al*. described that T-cells activated by anti-CD3 mAb and IL-2 were mainly CD4 cells that had both killer and helper functions (12). Administration of a CD20xCD3 BsAb (not BIS20x3) resulted in a cytokine profile in patients characterized by transient increases of IL-6, IL-8 and IL-10 (13). This suggests a more CD4-like response. Mack *et al*. described more in detail that *in vitro* activation of T-cells and target cell lysis via an anti-CD3 bispecific single chain Ab occurred at 4 hours for CD8-positive cytotoxic T-cells, whereas CD4-positive T-cell effector cells needed over 20 hours to reach similar levels of cytotoxicity (14). This was explained by the presence of pre-existing lytic tools in CD8-positive cells (cytolytic granules and vesicle stored FasL (15)) as compared to the prolonged activation period CD4-positive T-cells need to acquire a cytotoxic phenotype (12). We showed (**chapter 2 and 3**) that T-cell mediated lysis via BIS20x3 was rapid, with maximal lysis occurring after two hours, suggesting a possible CD8-type response. However, in **chapter 6** we also showed that T-cell activation and expansion was maximal after 48-72 hours, suggesting activation of a relatively slow, CD4-like response mechanism. Probably, anti-CD3 targeting BsAbs, like BIS20x3, can activate both CD8- and CD4-like activation cascades, resulting in efficient rapid B-cell lysis, but also in gradual T-cell expansion and activation.

The interaction with MHC.

In **chapter 5**, we demonstrated a functional interaction between CD20 and MHC-class II molecules. CD20 and MHC class II colocalization and bilateral signaling may create a signaling platform where costimulatory molecules can offer optimal activation of adjacent T-cells. CD20 was previously described to be present and functionally active in such a platform (16). Barcia and colleagues observed that B-cells and T-cells could form immunological synapses after viral infection, demonstrating CD3 cluster formation and, interestingly, also CD20 segregation at the interface of these synapses (17). Cytolytic CD8 TcR/MHC class I synapses have previously been described to play a role in BsAb mediated tumour cell depletion (18). Zocher *et al* showed that lytic synapses formed via single chain BsAbs displayed a composition and distribution pattern indistinguishable from the human T-cell cytolytic synapse, containing CD3, LFA-1, CD45, tyrosine kinase Lck, CD2, and several actin cytoskeleton related proteins (18). We suggest that simultaneous

binding of a BsAb (e.g. BIS20x3) to B-cells and T-cells, can influence formation of TcR/MHC-like synapses, inducing multiple types of cascades of T-cell activation and lysis. HLA-DR signaling within a MHC class II platform is suggested to be stabilized through CD20 signaling. The interaction with HLA-DR appears to depend on the capacity of CD20 to translocate to lipid rafts. We think BIS20x3 activation of T-cells may induce an interaction between the TcR-complex on the T-cell and MHC class II molecules on the B-cell since they appear present in the same signaling platform as CD20. This interaction may stabilize and induce a process mimicking normal antigen recognition and induction of costimulation, similar to regular immune synapse function.

The role of Fas signaling.
Mack *et al* described that addition of blocking Fas-Abs caused a reduction of lysis induced via bispecific anti-tumour / anti-CD3 BsAbs, suggesting a role for the death receptor pathway as T-cell effector mechanism (14). Addition of TNF-α or INF-γ blocking Abs did not reduce the rate of specific lysis. T cells can also up-regulate Fas expression themselves upon BsAb stimulation (19,20). This upregulation of Fas is in consensus with the theory of the activation-induced cell death (AICD) of cytotoxic T-cells upon target-cell recognition, limiting dangerous (auto)immune responses. Costimulation of T-cells during target-cell recognition could overcome this type of apoptosis induction and is therefore very important for control of BsAb / T-cell mediated immunotherapy (20,21).

However, although Fas-mediated cascades appear relevant for BsAb effectivity *in vitro, in vivo* many B-cell lymphomas express mutated forms of Fas to circumvent apoptosis. These mutations are suggested to be involved in the pathogenesis of lymphoproliferative diseases and autoimmune processes. Fas mutations were described in 10-20% of germinal center or non-germinal center NHLs, especially in DLBCLs and low-grade MALT-type lymphomas (22-24). Resistance within the Fas / death receptor pathway may help tumour cells to avoid being killed by cytolytic T-cells. It can also permit tumour cells to express FasL on their surface without killing themselves via an autocrine Fas/Fas-L mechanism (25). This FasL "counterattack" is responsible for tumour-induced killing of immune cells, destroying activated helper T-cells. Different mechanisms for resistance to apoptosis induction by Fas (or other death receptors) can include: 1) downregulation of the receptors, 2) inactivating mutations within the Fas gene, 3) increased expression of soluble or decoy receptors that compete for TNF family ligands and 4) overexpression of anti-apoptotic proteins, such as Bcl-2 or Bcl-xL (26,27). These resistance mechanisms may hamper the effectiveness of (non-CD20) BsAb-mediated T-cell apoptosis induction in tumour cells.

However, Vega *et al* demonstrated that CD20 targeting via rituximab could overcome Fas-resistance resulting in the sensitization of NHL cells to Fas-induced apoptosis. They showed that rituximab treatment resulted in inhibition of p38MAPK and NF-κB signaling, resulting in inhibition of the transcription repressor Yin-Yang 1 and subsequent upregulation of Fas expression on the B-cells (26,28). They also showed that this was a direct effect of the antibody binding to its cognate CD20 receptor, and did not require the Fc-binding domain of rituximab (29). In **chapter 4** we demonstrated that Ab-binding to CD20 creates a spontaneous clustering of Fas molecules on the surface of target B-cells and sensitizes them to Fas-induced apoptosis. Not only the upregulation of Fas expression (as shown by Vega *et al*) but also receptor independent multimerization, recruitment of caspase-8 and formation of the DISC was observed, upon CD20 triggering. This CD20-specific cascade is likely to decrease the effect of Fas-resistance mechanisms and make the target B-cells more prone to T-cell mediated lysis. The above described characteristics of CD20 signaling may provide a tool for BIS20x3 to circumvent the resistance of NHL cells to death-receptor mediated apoptosis. Several studies performed by our group and others have shown that combinations of CD20 and death-receptor-targeting-Abs induce synergistic levels of cell death in B-cells as compared to single mAbs alone, also suggesting intracellular mechanisms by which CD20 signaling can effect Fas-apoptosis sensitivity and vice versa (30-32). Therefore, the choice for CD20 as the target of an anti-CD3 BsAb appears to be especially favourable since it can induce apoptosis by CD20-crosslinking itself (**chapter 2, 3 and 6**), it can sensitize B-cells to Fas-mediated apoptosis (**chapter 4**) and may decrease apoptosis resistance in tumour cells either via Fas-sensitization or other intracellular mechanisms, for example via NF-κB (**chapter 3** and (33)) . All of the above processes may eventually result in unexpected high levels of T-cell activation *in vivo* and they may be clinically relevant in terms of the efficiency of B-cell depletion in patients.

CD20 and the BcR.

As described extensively by Glennie *et al*, CD20 mAbs can be divided into type I (rituximab-like) and type II (tositumomab-like) Abs. Type I mAbs induce translocation of CD20 into lipid rafts and evoke complement activation and classical apoptosis, whereas type II mAbs induce homotypic adhesion and non-apoptotic cell death (34-36). BIS20x3 was originally derived from the mAb B-ly1, a typical type I anti-CD20 Ab. Most other anti-CD20 BsAb described in literature are also of this type, therefore capable of using type I characteristics for signaling purposes. In the last few years, a significant body of attention has been given to the interaction of CD20 and the BcR following CD20 ligation with type I anti-CD20 directed antibodies. It was suggested that both CD20 expression on the membrane of the

cell and its redistribution into lipid rafts was influenced by BcR engagement (37). However, colocalization of the BcR and CD20 in lipid rafts after receptor ligation was described to be rapidly followed by activation-dependent dissociation of the two into distinct lipid rafts (38). Pre-treatment of B-cells with rituximab resulted in inhibition of the BcR-signaling cascade involving Lyn, Syk, PLCγ2, Akt, and ERK, and calcium mobilization (39). Ab-induced CD20 signaling also correlated with decreased raft-associated cholesterol, inhibition of BcR translocation into lipid rafts, and down-regulation of BCR immunoglobulin expression. BcR-stimulated calcium influx appeared significantly reduced by downregulation of CD20 expression (40). It appears that several CD20- and BcR-signaling effects occur parallel, interact with or influence each other. It was proposed that CD20, similar as described for HLA-DR, may be part of a signaling platform surrounding the BcR and may use its signaling pathway to orchestrate downstream signals triggered by the BcR (39,41). This characteristic feature of CD20 to interact with major B-cell signaling complexes, like the BcR and MHC class II, may eventually reveal the true functional role of CD20 on the surface of B-cells and explain why no natural ligand for CD20 has been described thus far. It may very well be that, similar to other members of the MS4A-family and most tetraspanins, CD20 is a subunit of a larger signaling-complex to which signals, obtained via closely related complex partners as HLA-DR or the BCR, are conveyed and/or amplified.

CD20 and the germinal center reaction.

Recently it was shown for the first time that a CD20 -/- patient displayed reduction of class-switched memory B-cells, impaired transition of naïve B-cells into marginal zone B-cells and suboptimal germinal center formation (42). Based on the results described in this thesis, we suggest that the biological function of CD20 may relate to the germinal center reaction, which involves clonal expansion of B-cells via TcR/MHC class II interaction, BcR somatic hypermutation and high-affinity B-cell selection. CD20 may also be involved in the negative selection of low affinity B-cells and self-reactive B-cells, since it is known that Fas (and, possibly, other death receptors) plays a pivotal role in germinal center B-cell apoptosis. It was proposed that germinal center B-cells display a "spontaneous" aggregation of Fas, resulting in a pre-assembled DISC. This enables ligand independent induction of apoptosis through Fas (43). Maintenance of expression of FLIP in B-cell centroblasts locks the DISC in a non-functional conformation. This ensures that B-cell death is prevented during the clonal expansion and repertoire diversification phase in the germinal center. FLIP expression is sustained by ligation of the BcR and / or CD40 in the context of MHC-class-II-restricted Ag presentation (44). Low affinity B-cells appear unable to maintain stable FLIP expression. This will result in an operational DISC that leads to activation of caspase-8 and, eventually, promotes apoptosis (45). We

suggest that CD20 might be involved in inducing or sustaining a spontaneous aggregation of Fas molecules, creating a pre-assembled DISC necessary for negative selection of low-affinity or self reactive B-cells in the germinal center. The natural environment of CD20 may be the immunological platforms containing MHC class II molecules or the BcR, present on B-cells in the germinal center. CD20 may be functionally involved in early T-cell / MHC interaction, BcR organisation and spontaneous Fas clustering, all processes pivotal for B-cell development in the germinal center, therefore creating a ligand independent biological function of CD20 on B-cells.

Ten arguments in favour of BIS20x3-mediated B-cell depletion.

In this thesis we have covered several previously reported facts and newly demonstrated signaling mechanisms which, all together, provide evidence that BIS20x3 employs multiple effector-mechanisms to induce B-cell depletion in a highly efficient manner.

In summary: **1)** CD20 is highly expressed on normal B-cells and on over 90% of all non-Hodgkin lymphomas. **2)** CD20 is only expressed on B-cells during a number of stages of B-cell development, but is absent from B-cell progenitor cells and mature plasma cells, preventing severe immune suppression. **3)** CD20 is not internalized nor shed from the cell membrane upon Ab binding, sustaining BIS20x3 availability for T-cell binding. **4)** Binding to CD20 by BIS20x3 can induce apoptosis through CD20 crosslinking independently of T-cell lysis. **5)** T-cells are efficiently activated by BIS20x3, due to the costimulatory capacities of the target B-cells. **6)** These costimulatory capacities are specifically enhanced by CD20 signaling in the target B-cells. **7)** Translocation of CD20 to lipid rafts triggers Fas expression, aggregation, formation of the DISC and induction of apoptosis. **8)** Increased Fas expression and raft dependent Fas clustering enhances the susceptibility of target B-cells to T-cell mediated FasL ligation and apoptosis. **9)** CD20 physically and functionally interacts with HLA-DR, probably within the context of the MHC-class II immune synapse, creating a platform for related molecules necessary for efficient activation of the effector T-cells. **10)** CD20 signaling can increase the sensitivity of B-cells to apoptosis via a complex network of intracellular signals which can be divided in 3 categories: a) membrane-associated proximal events, including sphingolipid distribution alterations, modifications of lipid rafts and calcium fluxes, as well as release of potent second messengers, such as ceramide, b) the inhibition of downstream signaling pathways involving MAPK, phosphoinositide 3-kinase/Akt, some specific protein kinase C isoforms, ERK and NF-κB, c) the last category is composed of proteins controlled by these signaling modules, the Bcl-2 protein family being the most representative. The above 10 items together suggest a

strong rationale for further *in vivo* exploration of the possible clinical benefits of BIS20x3 in B-cell depletion therapy.

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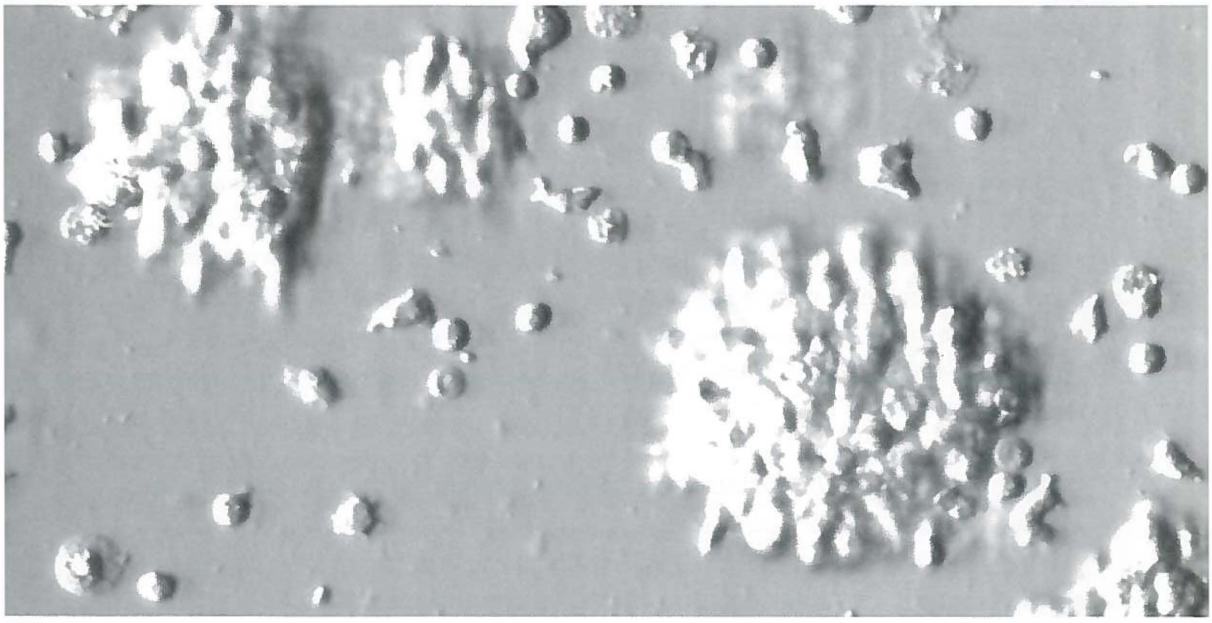
Chapter 9



BsAbs in the clinic and future perspectives.

BsAbs in the clinic and future perspectives.

The idea of a "magic bullet" for cancer therapy was first proposed by Paul Ehrlich. In 1908 he received the Nobel Prize in Medicine for his scientific work in the field of immunity. At the beginning of the 20th century he postulated that if a compound could be made selectively targeting a disease-causing organism, then a toxin could be delivered along with the agent of selectivity.

In 1975, scientists Kohler and Milstein developed the hybridoma technique for the production of monoclonal antibodies, a discovery also recognized by the award of the Nobel Prize in Medicine in 1984 (1). This led to an enormous expansion in the exploitation of antibodies in science and medicine. BsAbs, originally prepared by fusion of two myeloma cells, provided a new class of monoclonal antibodies, combining the specificities of two predefined monoclonal antibodies into one new Ab molecule. The first BsAbs described were used for immunocytochemistry and immunoassay purposes (2). It was soon thereafter that Staerz *et al* proposed to combine the power of effector T lymphocytes with the specificity of monoclonal antibodies to target predefined target cells (3). Engaging T cells for tumour cell elimination appeared a compelling concept because T cells are abundantly present in blood, lymph and various organs, are extremely motile and are equipped with an effective arsenal of mechanisms to kill target cells like granzymes and death receptors.

Initial clinical studies with BsAbs were rather disappointing mainly due to low efficacy, adverse effects such as fever, chills, bone pain, dyspnoea or allergic reactions and immunogenicity of the BsAbs. Among the first phase I clinical studies, performed using a BsAb (BIS-1), were studies executed by members of our group, directing T-cells to the pancarcinoma-associated antigen, EGP-2 / EpCAM (4,5). Conjugate formation between tumour cells and BIS-1 activated lymphocytes was observed upon local application of autologeous activated T-cells and BIS-1 in carcinoma patients. This was followed by a disappearance or reduction of tumour cells after 24-48 hours. Furthermore, an increase in local granulocyte numbers was observed in effusion fluids and also levels of IL-6 and TNF-α were significantly elevated. These data suggested that the treatment induces both anti-tumour activity and a strong local inflammatory reaction. Acute BIS-1-related toxicity with symptoms of chills, peripheral vasoconstriction and temporary dyspnoea was observed in patients treated systemically with BIS-1 (5). A rapid decrease was seen in the numbers of PBLs, monocytes and eosinophils. This was explained by the assumption that BIS-1-carrying T cells leave the circulation, induce high TNF-α production upon activation and thereby cause subsequent extravasation of

additional immune cells (6,7). However, whereas BsAb-directed anti-tumour activity could be demonstrated to be present in blood samples of these patients in an in *vitro* assay, no clear clinical responses were observed (8).

More promising clinical responses were reported in a combined Italian and Dutch BsAb study, targeting ovarian cancer cells using a BsAb (OC/TR) recognizing the folate-binding protein on ovarian cancer cells on the one hand and the CD3 activation site on T lymphocytes on the other, showing 5 complete remissions (CR), 3 partial regressions (PR), 2 stable disease (SD) and 3 progressive disease (PD) (9). However, all patients developed human anti-mouse-antibodies (HAMA). In a following study with the same BsAb, 26 patients with limited intraperitoneal disease after first-line therapy entered a phase II study. Seven out of 26 patients (27%) showed complete or partial intraperitoneal responses (10,11). Complete responses in three patients lasted respectively 26 months, 23 months and 18 months. However, in most patients, the disease relapsed outside the peritoneal cavity, and in one case complete intraperitoneal response was accompanied by progression in retroperitoneal lymph nodes.

In patients with metastatic breast cancer that overexpressed HER2/neu, a phase I study was conducted with a BsAb that binds to both HER2/neu and the FcgammaR1 receptor (CD64) (12). Similar to BIS-1, significant release of cytokines interleukin-6 and TNF-α was observed after administration. The toxicity of the BsAb was described as modest, including fevers, diarrhoea, and allergic reactions that did not limit therapy. Significant levels of HAMA were measured in the plasma of most patients. Unfortunately, with this BsAb, no objective clinical responses were seen in this group of heavily pre-treated patients.

The first studies targeting B-cells with BsAbs were performed with CD19-targeting Abs. One study within a small group of chemotherapy-resistant CLL patients showed limited toxicity administering the BsAb (CD3xCD19), but no beneficial clinical effects (13). In another study, locoregional treatment of B-cell lymphoma with CD3xCD19 BsAbs combined with CD28 costimulation resulted in a local response in only 2/9 (22%) patients (14). Five patients developed HAMA after injection and two patients developed mild to moderate adverse effects consisting of erythema and fever. In one out of three investigated patients an increased T-cell response toward autologous idiotype peptides was seen, suggesting that at least in some patients lymphoma-specific T-cells can be recruited by this immune-therapeutic approach toward B-cell lymphoma (15). In a group of 38 NHL patients (follicular lymphoma, mantle cell lymphoma, and chronic lymphocytic leukemia) treated with the single chain CD19xCD3 BsAb blinatumomab, 11 beneficial responses were observed (seven PR and four CR) (16). Longest duration of a CR

was 13 months in a patient with mantle cell lymphoma and three more patients had ongoing response lasting for more than six months. Blinatumomab also led to clearance of tumour cells from bone marrow and liver. *In vitro* blinatumomab consistently led to a higher degree of lysis of human lymphoma lines than rituximab, and was active at much lower concentrations (17).

In a pilot clinical trial with a CD20xCD3 targeting BsAb, Bi20, six patients (three cases with CLL and three with NHL) refractory to standard therapy were treated with escalating doses of Bi20 (range 10-2000 μg) followed by donor lymphocyte infusion (DLI) or stemcell transplantation (18). All CLL patients showed a prompt but transient clinical and hematological response. In one patient with NHL, a halt in progression was observed for almost 4 months. Side effects (fever, chills and bone pain) were tolerable. The cytokine profile was characterized by transient increases of IL-6, IL-8 and IL-10. Neither HAMA nor GVHD developed, allowing repeated treatment courses.

Although several BsAbs, especially BsAbs targeting B-cells were described to be relatively safe in patients and resulted in CR or PR in some patients, no large phase III clinical trials or randomized controlled trials have ever been performed with BsAbs. This is probably due to a combination of problems. First, adverse effects might be described modest, but the relatively unknown and uncontrolled activation of effector T-cells caused by high production of several cytokines, the rapid extravasation of T-cells and the unpredictable co-effects on other immune cells, may hamper further development of BsAb mediated T-cell retargeting therapies. For example in the blinatumomab study of Bargou *et al*, treatment was discontinued in 7/40 patients, partly because of symptoms of the central nervous system, such as confusion, disorientation, and speech disorder (16). Secondly, only a few studies show clinically relevant response rates in patients treated with BsAbs, although it has to be noted that most studies were small and not designed to demonstrate response rates or increased overall survival. Another relevant problem may be the high amount of HAMA responses developed in patients, because most trials were performed with murine BsAbs. The HAMA response is essentially a reaction to the mouse antibodies that can range from a mild form, like a rash, to a more extreme and life-threatening response, such as renal failure. HAMA can also decrease the effectiveness of the treatment, or increase the risk of a future reaction if the patient is given a subsequent treatment containing mouse antibodies. Probably, the humanization of BsAbs could circumvent this reaction and result in safer application of this type of Ab therapy.

In recent years, multiple new types of Abs, murine (-omab), chimeric (-iximab), humanized (-zumab) or completely human (-umab), have been investigated. Second and third generation anti-CD20 directed mAbs have been developed to overcome resistance to rituximab. Several studies have been performed or are still ongoing to show increased efficacy compared head-to-head with rituximab or to show effectiveness of these new reagents in rituximab-refractory NHL patients. In second generation reagents the IgG1 mAb is humanized or fully human to reduce immunogenicity. Third generation mAbs are also humanized, but additionaly have an engineered Fc region designed to improve therapeutic performance of the Ab by increased recruitment of immune effector components.

At least seven mAbs are now in clinical development, with many more in pre-clinical evaluation.

The type II murine Ab tositumomab (B1) is a murine IgG2a λ mAb. Ionising radiation therapy with covalently linked Iodine-131 to tositumomab is successfully used for the treatment of patients with follicular and transformed NHL who failed or relapsed from prior rituximab treatment and standard chemotherapy (19).

A second Ab, the humanised ocrelizumab is derived from the murine 2H7 anti-CD20 Ab and is a type I mAb with an IgG1 isotype. In a phase I/II clinical trial, ocrelizumab was administered to rituximab pretreated patients with relapsed/refractory follicular NHL. It was well tolerated and showed a response rate of 36% (20). Ocrelizumab in combination with methotrexate was studied in a phase I/II trial in the treatment of RA and showed minimal immunogenicity and improved B-cell depletion as compared to placebo (21). Currently, clinical effectiveness of ocrelizumab is studied in phase III clinical trials for RA and lupus nephritis, and phase II trials for multiple sclerosis.

In 2009 ofatumumab was approved by the FDA for the treatment of patients with CLL refractory to fludarabine and alemtuzumab (22). Ofatumumab binds the small extracellular loop of the human CD20 molecule, which is in a closer proximity to the cell membrane than the binding site of rituximab, which binds to the larger loop. This is suggested to be the most important reason why ofatumumab is more potent than rituximab in inducing complement-mediated lysis (23,24). In patients with refractory CLL overall response rates were achieved up to 58% upon treatment with ofatumumab. Median progression-free survival and overall survival times were 5.7 and 13.7 months (25).

A fourth Ab, veltuzumab, is a type I, anti-CD20 directed humanised IgG1 MAb generated by using the complementary determining regions (CDR) from the parental murine A20. The first clinical studies have shown favourable safety and efficacy results in NHL patients with lower doses and less administrations of Ab, achieving overall response rate up to 44% (26). Also a phase I study is ongoing for the treatment of immune thrombocytopenic purpura.

Third generation anti-CD20 mAbs currently in development are TRU-015, AME133V, Pro13192 and GA101 (27-30). The latter is the only new Ab of the type II group, generated by grafting the CDR sequences of the B-ly1 anti-CD20 mAb (a classical type I mAb and the parental mAb used for our BIS20x3) on a framework regions of fully human IgG1-kappa. In addition, the Fc region has been glycoengineered, which resulted in a 50-fold higher affinity to human FcγRIII receptors (31,32). It appeared that by recombinant engineering the Abs characteristics were altered from a classical type I to the more rare type II. Up to today it is not known how or what has caused this alteration. Apparently structural modifications of the Ab can alter its functionality, changing it from a type I to type II.

The last few years, anti-CD20 Abs are increasingly used in treatment of auto-immune diseases. In these chronic inflammatory diseases, high or aspecific cytokine responses and aberrant cellular responses are abundantly present with active disease. For example, B-cell mediated T-cell costimulation has been described to be pathophysiologically important for the development of rheumatoid arthritis or SLE (33,34). B-cell depletion by rituximab was suggested to decrease unwanted T-cell costimulation, and also resulted in correction of in aberrant expression of costimulatory molecules in patients with active disease (35).

BIS20x3 however, may have an opposite effect and unwanted effect in auto-immune diseases as compared to rituximab. It induces costimulatory interactions between B-cells and T-cells, resulting in a severe increase in T-cell activation and secondary B-cell depletion. An uncontrolled activation of T-cells however, may increase auto-immune disease activity in stead of decreasing it. This may make it difficult for BIS20x3 to be safely administered in auto-immune diseases. Therefore, BIS20x3 or human(ized) BIS20x3, may not be not feasible for further clinical approach in auto-immune diseases. Currently, other alternative therapeutical options are present and more experience should first be obtained by the use of regular anti-CD20 Abs in auto-immune diseases.

Taken into consideration the data that are shown within this thesis and the above described facts and findings, we suggest additional experiments should be performed for the development of BIS20x3 as a therapeutic tool. It appears important to investigate whether BIS20x3 displays type I properties, like its parental anti-CD20 Ab, or if structural Ab changes may have changed the anti-CD20 Ab type. Additionally, it may by necessary to modify BIS20x3 into a humanized or completely human BsAb. This may circumvent the problem of high levels of HAMA production caused by murine BsAbs and could even increase clinical effectivity since a human Fc-domain would then also be available for ADCC and CDC mediated lysis. However, uncontrolled immune responses with interactions between highly activated T-cells, macrophages and complement, resulting in extreme bursts of cytokine release might have to be expected. Therefore a phase I and/or II trial should first be performed to assess drug safety, dosing requirements and drug efficacy. In patients with refractory B-cell lymphoma, resistant to standard therapy or to the above mentioned new anti-CD20 therapeutics, it may be justified to perform such a study, observing the possible potential of a human(ized) BIS20x3. High T-cell activation responses and levels of cytokine release should be expected and patients should be under careful in-hospital control during and post-infusion.

In summary, many BsAbs have been tested in clinical trials. However, the results with these Abs have been relatively disappointing so far. Clinical response appears limited and adverse effects, although often reported minor, are unpredictable. No large phase III or IV trials have been performed with BsAbs so far. However, targeting B-cells with an anti-CD20 BsAb, such as BIS20x3, may still be promising since it can use the costimulation capacities of the B-cell for increased T-cell activation. Secondly BIS20x3 can benefit form CD20 signaling effects resulting in increased sensitivity to apoptosis induced by simultaneous applied chemotherapeutics. Thirdly, sensitization to Fas-mediated cell death increases T-cell mediated apoptosis in B-cell targets. Before BIS20x3 can be used in patients, it probably should be humanized to avoid HAMA responses and increase additional immune responses like ADCC and complement-mediated lysis. We believe that clinical application of a humanized BIS20x3 may be successful in refractory B-cell lymphoma, although under careful control of vital functions since high levels of T-cell activation and uncontrolled cytokine release with systemic effects may be expected.

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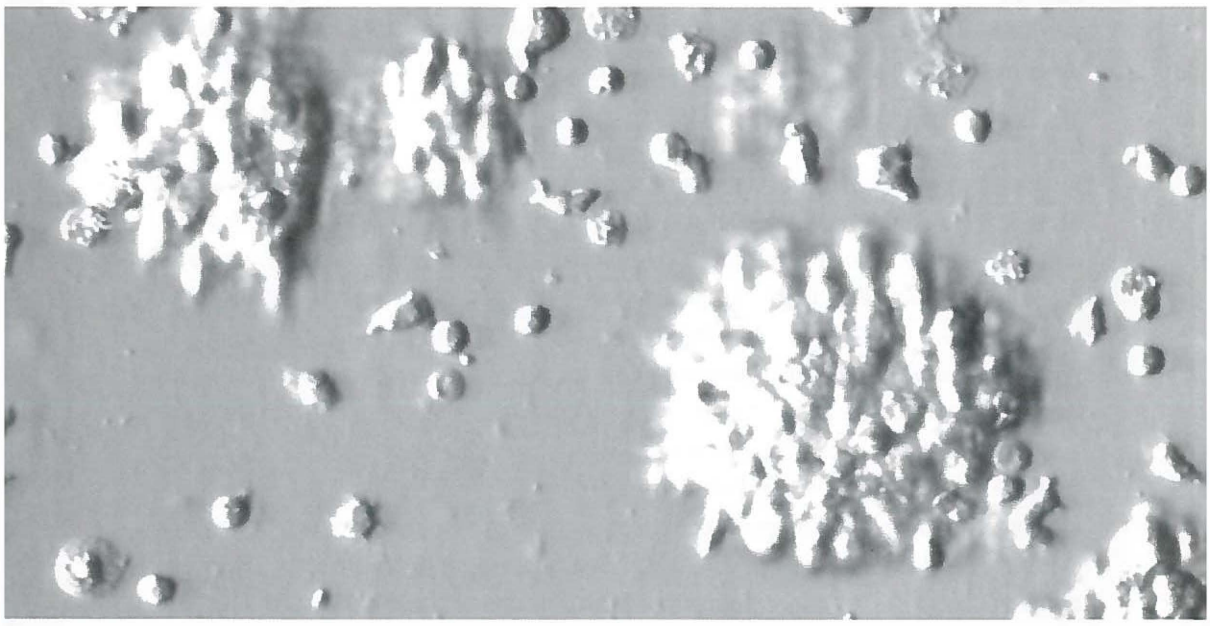
Chapter 10



Nederlandse samenvatting voor niet-ingewijden.

Nederlandse samenvatting voor niet-ingewijden.

Immunologische afweer.

Lymfocyten zijn witte bloedcellen die een belangrijke rol spelen in ons afweersysteem. Ze zijn vooral belangrijk bij de afweer tegen lichaamsvreemde stoffen, zoals bacteriën en virussen. Er kan onderscheid gemaakt worden tussen T-lymfocyten en B-lymfocyten, ook wel T- en B-cellen genoemd. Lymfocyten worden continu aangemaakt door stamcellen en kunnen vervolgens uitrijpen tot volwassen B- en T-cellen. Voor T-cellen gebeurt dit in de thymus (zwezerik), vandaar de naam T-lymfocyt. De naam B-lymfocyt is oorspronkelijk afkomstig van de Bursa van Fabricius. Dit is een orgaan dat alleen bij vogels voorkomt en waar de B-cellen in uitrijpen. Bij mensen en andere zoogdieren gebeurt dit niet in één enkel orgaan, maar in o.a. de lever en vooral het beenmerg.

B-cellen

B-cellen zijn de cellen die verantwoordelijk zijn voor de productie van antilichamen. Tijdens de uitrijping van de B-cel treedt een herschikking van DNA op (DNA bevat de code waarin al onze erfelijke eigenschappen zijn vastgelegd), waardoor elke B-cel codeert voor één uniek antilichaam (immuunglobuline of Ig). Antilichamen kunnen een specifieke binding aangaan met antigenen. Dit zijn de herkenningseiwitten van lichaamsvreemde stoffen. Wanneer ergens een antigeen beschikbaar is, zal de best "passende" B-cel binden aan het antigeen. De B-cel die het antigeen herkent, wordt tot deling gestimuleerd. Hiervoor heeft de B-cel hulp (costimulatie) nodig van zogeheten T-helper cellen. De B-cellen rijpen vervolgens verder uit en er ontstaan vele B-cellen (plasmacellen) die grote hoeveelheden van dezelfde antilichamen maken. Er worden ook B-geheugencellen (memory B-cellen) aangemaakt. Deze zorgen ervoor dat er tot jaren na een eerste contact met een bepaald antigeen steeds opnieuw een specifieke en snelle immuunreactie kan optreden. De ontwikkeling van memory B-cellen gebeurt in de secundaire lymfoïde organen als de milt en lymfeklieren. In deze organen bevinden zich de zogenoemde kiemcentra (germinal centers) waarin B-cellen zich kunnen differentiëren naar specifieke antilichaamproducerende B-cellen met een steeds hogere affiniteit voor het aangeboden antigeen. Daarnaast wordt hier de productie van memory B-cellen gereguleerd. Deze centra spelen dus een centrale rol in de ontwikkeling van het B-cel repertoire van een individu.

Immuunglobulines.

Antilichamen, ofwel immuunglobulines, kunnen worden onderverdeeld in verschillende klassen zoals IgG, IgA, IgM, IgD en IgE. De basisstructuur van een IgG immuunglobuline bestaat uit twee lange of "zware" ketens en twee korte of

"lichte" ketens. Per lichte en zware keten is er slechts een klein gedeelte dat variabel is in samenstelling. Dit variabele gedeelte is uniek waardoor het immunoglobuline in staat is specifiek te reageren met één antigeen. Hiernaast heeft elke immuunglobuline een constant gedeelte, dat het Fc-domein wordt genoemd. Dit Fc-domein kan een interactie aangaan met andere componenten van het immuunsysteem zoals "complementfactoren" die via een kettingreactie (cascade) aanleiding geven tot het lek en kapot gaan van de met immuunglobulines bedekte cellen. Dit proces heet "complement dependent cytotoxicity". Anderzijds kan het Fc-domein binden aan Fc-receptor positieve cellen (zoals "natural killer" NK cellen of macrofagen). Hierdoor komt vervolgens een proces op gang waarbij bacteriën of cellen opgenomen en verteerd kunnen worden (fagocytose) in combinatie met de productie van diverse toxische stoffen. Dit proces wordt ADCC ("antibody dependent cellular cytotoxicity") genoemd.

T-cellen

Er kunnen verschillende typen T-cellen met verschillende functies onderscheiden worden.

T-helpercellen (kortweg T_h-cellen) zijn ondersteunende T-cellen die verschillende soorten immuunregulerende cytokines (interleukines) afgeven. Onder invloed van bepaalde cytokines ontwikkelen B-lymfocyten zich tot antilichaam-producerende plasmacellen. De interactie tussen B-cellen en T_h-cellen in de kiemcentra is essentieel voor de positieve selectie van B-cellen die antistoffen produceren met een hoge affiniteit. Hiernaast zorgen T_h-cellen er voor dat B-cellen die onvoldoende specifiek of zelfreactief zijn, opgeruimd worden uit de populatie.

Cytotoxische T-cellen (T_c-cellen) zijn "killer'' T-cellen die zorgen voor de eliminatie van geïnfecteerde, beschadigde of dysfunctionele cellen. Zij doen dit door toxische stoffen te produceren die de te elimineren cellen perforeren en doden. Hiernaast kan celdood geïnduceerd worden door interacties tussen oppervlakte-eiwitten op de T-cel en de geïnfecteerde cel. Deze oppervlakte-eiwitten kunnen binden aan zogenoemde "death-receptoren" op de geïnfecteerde cel, zoals bijvoorbeeld Fas-ligand (FasL) dat bindt aan Fas. Door deze binding wordt een reeks aan eiwitreacties geactiveerd waardoor de geïnfecteerde cel op een gecontroleerde manier sterft. Dit proces heet geprogrammeerde celdood of apoptose.

Op T-cellen komt ook een celspecifieke receptor voor, de T-cel receptor of TcR. Deze bevindt zich in een complex (het TcR complex) met andere moleculen. CD3 is een van de belangrijkste TcR geassocieerde eiwitten en verzorgt voornamelijk het doorgeven van T-cel activatie signalen naar de kern van de cel. T-lymfocyten

worden pas gestimuleerd wanneer ze binden aan een antigeen dat gepresenteerd wordt in een bepaald complex (het major histocompatibility complex ofwel MHC-complex). Dit kan o.a. worden gedaan door specifieke antigeen presenterende cellen (bijv. macrofagen, dendritische cellen, maar ook B-cellen). Het TcR complex reageert met een specifiek antigeen, gebonden aan het MHC-complex op de antigeen presenterende cel. Het signaal van het TcR complex wordt vervolgens versterkt door de gelijktijdige binding van de MHC moleculen met een specifieke co-receptor. Op T_c-cellen is dit CD8, dat specifiek is voor MHC klasse I moleculen. Op T_h-cellen is dit CD4, dat exclusief bindt aan MHC klasse II. Tenslotte is er voor optimale T-cel avtivatie nog een extra signaal nodig (costimulatie). Hiervoor treedt er binding op tussen costimulatoire moleculen op het oppervlak van beide celtypen, zoals tussen CD28 op de T cel en CD80/CD86 op een antigeen presenterende cel. Bij afwezigheid van dit laatste signaal wordt de T-cel niet geactiveerd en gaat zelf in apoptose.

Behandeling van B-cel gemedieerde ziekten.

Wanneer de negatieve selectie voor aspecifieke B-cellen onvoldoende plaatsvindt, kan het voorkomen dat ongewenste, defecte B-cellen ongeremd gaan uitgroeien. Dit kan leiden tot de ontwikkeling van vormen van kanker uitgaande van lymfocyten; maligne lymfomen. Hiernaast kunnen zelfreactieve B-cellen ontstaan die een immuunreactie tegen “eigenlichaam”-eiwitten opwekken (een auto-immuun reactie), resulterend in diverse chronische inflammatoire aandoeningen als reumatoïde artritis, SLE of de ziekte van Sjögren. Zowel B-cel lymfomen als B-cel gemedieëerde auto-immuunziekten zijn ernstige aandoeningen die moeilijk te behandelen zijn. Al jaren wordt gezocht naar B-cel specifieke eiwitten die gebruikt kunnen worden als doelwit voor therapieën die zich specifiek richten op het elimineren van (zieke) B-cellen. Sinds 1997 worden B-cel lymfomen behandeld met rituximab, vaak in combinatie met chemotherapie. Rituximab is een antilichaam dat als antigeen CD20 herkent, een eiwit dat alleen op B-cellen in ontwikkeling voorkomt. Het is niet aanwezig op stamcellen of plasmacellen, zodat na behandeling de B-cel populatie weer normaal kan uitgroeien. Rituximab heeft een humaan Fc-domein, waardoor het, net als “normale” antilichamen, diverse onderdelen van het immuunsysteem kan activeren, zoals het complement systeem en ADCC. Hierdoor worden CD20-positieve B-cellen specifiek opgeruimd door activatie van het eigen immuunsysteem. Er is ook ontdekt dat binding van rituximab aan CD20 leidt tot het dicht bij elkaar brengen van meerdere CD20 moleculen op het oppervlak van de B-cel, een proces dat crosslinking wordt genoemd. Crosslinking van CD20 leidt tot de inductie van apoptose in de B-cel. De afgelopen 15 jaar is behandeling met rituximab erg succesvol gebleken en de laatste jaren wordt rituximab ook toegepast bij diverse auto-immuun ziekten, zoals

reumatoïde artritis en de ziekte van Sjögren. Het blijkt echter dat sommige lymfomen ongevoelig zijn voor rituximab behandeling, of na initieel goed te hebben gereageerd op rituximab, toch terugkeren. Om deze ongevoeligheid te omzeilen worden steeds nieuwe strategieën bedacht om B-cellen op een andere manier op te ruimen. Voorbeelden hiervan zijn antilichamen tegen een ander doeleiwit als bijvoorbeeld CD19 of CD52 (Campath), antilichamen tegen CD20 gelabeld met een radioactieve stof (Bexxar/Zevalin), of de productie van een volledig humaan anti-CD20 antilichaam (Ofatumumab/HuMax-CD20). De effectiviteit van deze middelen wordt op dit moment onderzocht in diverse klinische trials.

Bispecifieke antilichamen

Een van de mogelijke nieuwe strategieën voor behandeling van maligne lymfomen is de ontwikkeling van bispecifieke antilichamen. Dit zijn antilichamen die in staat zijn om met één antilichaam te binden aan twee verschillende antigenen. Enerzijds kan het een tumorspecifiek eiwit herkennen (tumorantigeen), maar met het andere deel kan het bijvoorbeeld een effector cel, zoals een T-cel, activeren. Bispecifieke antilichamen worden geproduceerd door twee antilichaamproducerende cellen te laten fuseren. Deze gefuseerde cellen maken zowel de beide originele ("ouder") antilichamen als een combinatieproduct dat zowel de ene arm van de ene "ouder" heeft en de andere arm van de andere "ouder". Het bispecifieke antilichaam kan vervolgens geïsoleerd en gezuiverd worden. De werking van het bispecifieke antilichaam bestaat uit de gelijktijdige binding van het bispecifieke antilichaam aan zowel een effector cel als aan het tumorantigeen, waardoor het leidt tot de activatie van de effector cel en het redigeren van de effector cel naar de tumorcel. In het geval van bijvoorbeeld een T-cel als effector cel wordt deze geactiveerd en worden de verschillende cytotoxische effecten van de T-cel specifiek gericht tegen de tumorcel. Al in 1983 werden bispecifieke antilichamen voor het eerst beschreven. Sindsdien zijn er diverse tumorantigenen onderzocht als "target" voor bispecifieke antilichaam therapie waaronder CD19 en CD20 op B-cellen. Vooral CD20 lijkt een geschikt tumorantigeen omdat het, zoals eerder besproken, alleen op vroege B-cellen voorkomt en via CD20 clustering zelfstandig apoptose induceert. Verder blijkt CD20, in tegenstelling tot bijvoorbeeld CD19, niet te internaliseren. Internalisatie houdt in dat het antigeen, na binding van het antilichaam, opgenomen wordt in de cel en dus niet langer aanwezig blijft op het oppervlak van de cel. Hierdoor kan de effector cel zijn doel niet bereiken, waardoor de effectiviteit van het bispecifieke antilichaam afneemt. In tegenstelling tot CD19 blijft CD20 continu beschikbaar op het oppervlak van de B-cel en is het bispecifiek antilichaam en vervolgens de B-cel optimaal bereikbaar voor de effector cel.

CD20

CD20 is een eiwit dat specifiek op het oppervlak van B-cellen voorkomt. CD20 zit stevig verankerd in het membraan doordat het meerdere keren door het membraan geweven zit. Door deze vouwing heeft het CD20 molecuul twee extracellulaire onderdelen (epitopen). Antilichamen, zoals rituximab, kunnen specifiek binden aan één van deze epitopen, maar een natuurlijk bindingseiwit (ligand) voor CD20 is tot op heden nooit ontdekt. Ook de fysiologische rol van CD20 op B-cellen is nog grotendeels onbekend, ondanks dat er uitgebreid onderzoek is gedaan naar de structuur en functie van CD20. Men heeft beschreven dat CD20 een calciumkanaal zou kunnen vormen of in ieder geval betrokken is bij calciumafhankelijke processen in B-cellen. Hiernaast zou CD20 betrokken kunnen zijn bij het verplaatsen van moleculen over het membraan naar specifieke lipidenplatforms (lipid rafts). Binnen deze lipid rafts komen CD20 en allerlei andere eiwitten samen, waardoor signalen van buiten de cel doorgegeven kunnen worden naar de kern van de cel. Voorbeelden hiervan zijn de activatie van tyrosine kinases, Src-kinasen, MAP-kinases, NF-κB etc. Het gevolg van deze activatie is dat bepaalde celprocessen als celdeling, differentiatie en celdood beïnvloed kunnen worden door CD20 signalering. Zo blijkt binding en crosslinking van CD20 eiwitten rechtstreeks apoptose te kunnen induceren via de activatie van specifieke apoptose-regulatie enzymen, de caspases. Ook andere pro- en anti-apoptotische eiwitten, zoals eiwitten van de Bcl-2 familie, kunnen in hun werking door CD20 worden beïnvloed. Tenslotte blijken ook centrale B-cel eiwitten als de B-cel receptor en MHC klasse II moleculen in directe relatie te staan met CD20. CD20 en het MHC klasse II molecuul HLA-DR zouden in direct fysiek contact met elkaar staan in lipid rafts en beide induceren vergelijkbare intracellulaire signalingscascades. Ook de B-cel receptor zou in bepaalde omstandigheden in direct contact staan met CD20.

Dit proefschrift

In dit proefschrift wordt een nieuw antilichaam beschreven dat mogelijk een verbetering zou kunnen zijn t.o.v. de huidige B-cel gerichte therapieën. Binnen ons laboratorium is een bispecifiek antilichaam ontwikkeld dat enerzijds, net als rituximab, bindt aan CD20 op B-cellen, maar anderzijds kan binden aan CD3 op T-cellen. Dit antilichaam wordt BIS20x3 genoemd en is in staat T-cellen te activeren via CD3 binding en vervolgens deze geactiveerde T-cellen te herrichten naar de "zieke" B-cel. In **hoofdstuk 2** hebben we laten zien dat BIS20x3 specifiek bindt aan CD20 op B-cellen en aan CD3ε, een onderdeel van de T-cel receptor. BIS20x3 bleek vervolgens een eiwitspecifieke activatie te induceren via deze beide moleculen. Hiernaast bleek BIS20x3 in staat T-cellen zeer efficiënt te activeren en vervolgens te

redigeren naar CD20-positieve B-cellen, met als gevolg dat deze B-cellen via een cytotoxische T-cel reactie gedood werden.

In **hoofdstuk 3** is dit principe verder uitgewerkt en is aangetoond dat de opvallende effectiviteit van BIS20x3 in het activeren van T-cellen veroorzaakt wordt door het feit dat B- en T-cellen elkaar, net als in de normale immuunreactie, kunnen activeren door het proces van costimulatie. Na binding van BIS20x3 aan CD20 op de B-cel werd bijvoorbeeld de expressie van het costimulatoire molecuul ICAM-1 hoger, een proces dat afhankelijk bleek van NF-κB signaling (net als veel andere constimulatoire reacties). Zowel het blokkeren van de binding van ICAM-1 aan zijn receptor LFA-1 als het onderdrukken van NF-κB in B-cellen bleek de mate van activatie van de T-cellen door BIS20x3 te verminderen. Hiernaast bleek BIS20x3 ook een veel efficiëntere activatie van T-cellen te geven wanneer dit vergeleken werd met een CD19xCD3 bispecifiek antilichaam. Blijkbaar zijn de signalen die geïnduceerd worden door BIS20x3 binding aan B-cellen specifiek betrokken bij het vermogen tot costimulatie, waardoor een optimale T-cel activatie plaats kan vinden. Een snelle BIS20x3 specifieke lysis van B-cellen door de geactiveerde T-cellen is hiervan het gevolg.

Binding van een antilichaam als BIS20x3 of rituximab aan CD20 leidt tot clustering van CD20 moleculen in de eerder genoemde lipid rafts. CD20 translocatie naar lipid rafts blijkt echter vergezeld te gaan met de gelijktijdige verplaatsing van andere moleculen naar lipid rafts. In **hoofdstuk 4** laten we zien dat binding van rituximab aan CD20 op B-cellen tot resultaat heeft dat ook de death-receptor Fas spontaan clustert in lipid rafts en vervolgens een death-receptor specifieke apoptose-cascade in gang zet. In dit hoofdstuk beschrijven we voor het eerst dat de death-receptor apoptose route, geïnduceerd via caspase 8, een rol speelt bij rituximab geïnduceerde B-celdood. Het mechanisme hiervoor lijkt te berusten op een, door CD20 geïnduceerde, clustering van Fas in rafts, gevolgd door de vorming van een speciaal death-receptor platform (death inducing signaling complex of DISC), activatie van caspase 8 en uiteindelijk inductie van apoptose. Dit mechanisme kan een verklaring zijn voor het feit dat B-cellen gevoeliger worden voor Fas-geïnduceerde apoptose wanneer deze gecombineerd wordt met rituximab behandeling.

In **hoofdstuk 5** wordt een ander molecuul geanalyseerd waarvan de interactie met CD20 in lipid rafts beschreven wordt. Dit betreft HLA-DR, één van de MHC klasse II moleculen. In dit hoofdstuk laten we zien dat HLA-DR en CD20 signalering volledig identiek lijkt te zijn. Wanneer HLA-DR ontbreekt op een B-cel bleek rituximab geen apoptose te kunnen induceren via CD20. Re-introductie van HLA-DR in deze zelfde

cellijn zorgde ervoor dat de gevoeligheid voor CD20-geinduceerde apoptose weer herstelde. Het lijkt dat CD20 signaling in ieder geval deels afhankelijk is van functionele HLA-DR expressie. In het verleden is al eens aangetoond dat CD20 en HLA-DR ook een rechtstreekse verbinding aangaan in rafts. Wij hebben laten zien dat, wanneer HLA-DR moleculen aangezet werden tot translocatie naar deze rafts, CD20 moleculen vanzelf volgden, zonder dat hiervoor binding van een anti-CD20 antilichaam nodig was. CD20 speelt dus mogelijk een rol in de opmaak van signalering via HLA-DR (de immuunsynaps) en is daarmee in staat om signalen, gegenereerd via MHC-klasse II moleculen, via hergroepering van membraan-processen door te geven naar de kern van de cel.

Bovenstaande hoofdstukken hebben ons geleerd dat BIS20x3 op een zeer efficiënte manier in staat is T-cellen te activeren en redigeren naar CD20-positieve B-cellen. Additionele costimulatie blijkt niet nodig omdat de B-cel dit zelf, en op een CD20-specifieke wijze, verzorgt. CD20-binding en stimulatie maakt B-cellen ook gevoeliger voor Fas-geinduceerde apoptose. Aangezien Fas-FasL binding één van de meest gebruikte cytotoxische mechanismen van geactiveerde T-cellen is, lijkt dit specifieke gevolg van CD20 stimulatie zeer gunstig voor BIS20x3 effectiviteit. Tenslotte blijken de signaleringroutes van CD20 en HLA-DR vrijwel identiek te zijn en is er ook een functionele interactie tussen beide moleculen. Dit wekt de suggestie dat de fysiologische rol van CD20 hier wel eens gevonden zou kunnen worden, waarbij CD20 mogelijk een ondersteunende functie heeft in de immuunsynaps. Het feit dat CD20 een natuurlijke plaats zou hebben in het contact tussen B-cellen en T-cellen kan uiteindelijk zeer gunstig zijn voor BIS20x3. Het zou verklaren waarom CD20 invloed lijkt te hebben op de costimulatoire capaciteiten van B-cellen en waarom T-cel activatie via BIS20x3 zo buitengewoon efficiënt lijkt te zijn.

In **hoofdstuk 6** laten we tenslotte zien dat BIS20x3 niet alleen effectief is in het redigeren van T-cellen naar B-cellen *in vitro*, maar ook effectief blijkt te zijn in een *in vivo* setting. In speciale muizen, die hun eigen immuunsysteem missen werd eerst een humaan B-cel lymfoom geïntroduceerd. Vervolgens werden humane T-cellen geïnjecteerd in deze muizen, wel of niet in combinatie met BIS20x3. Het bleek dat het aantal maligne B-cellen lager was in muizen die behandeld waren met BIS20x3 en dat daarnaast de overleving beter was in deze muizen. Ondanks dat er slechts een kleine groep muizen behandeld is, lijken de eerste resultaten in een *in vivo* setting dus hoopgevend voor verdere ontwikkeling van het bispecifieke antilichaam BIS20x3.

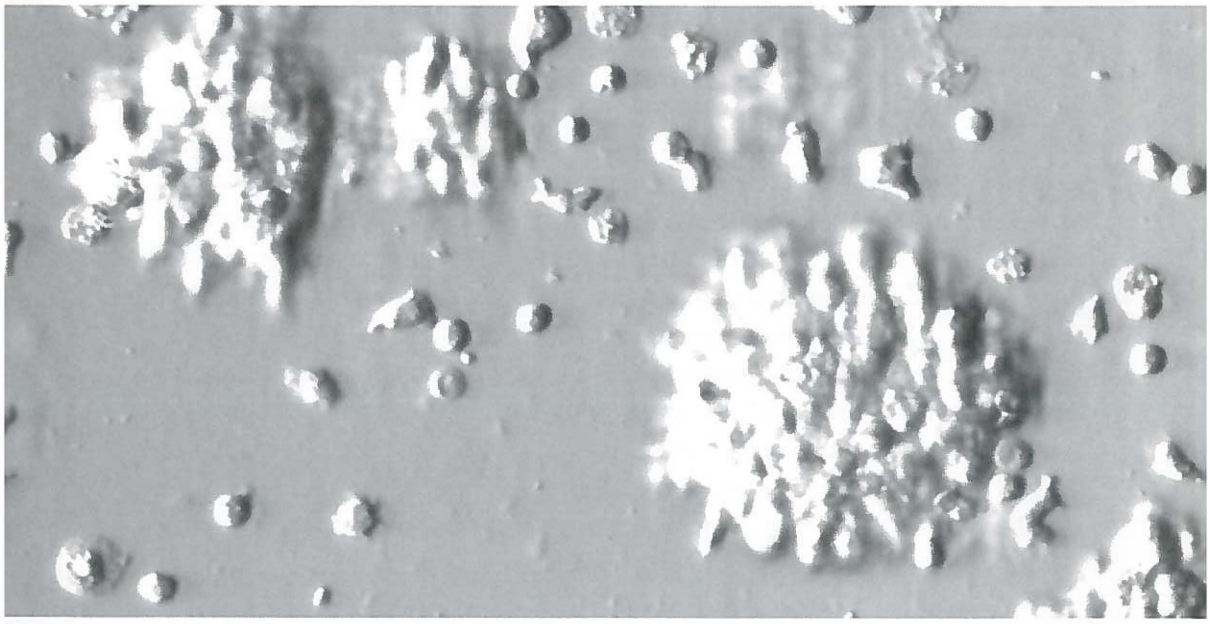
Discussie en verder onderzoek.

BIS20x3 lijkt een nieuw middel te kunnen worden in de behandeling van B-cel lymfomen en eventueel zelfs tegen B-cel gemedieerde auto-immuun aandoeningen. In dit proefschrift hebben we verschillende bekende en nieuwe eigenschappen van BSI20x3 en CD20 op een rij gezet die ondersteunen dat BIS20x3 een potentieel effectief nieuw medicijn kan zijn; 1) CD20 wordt niet geïnternaliseerd wanneer een antilichaam aan het eiwit bindt en blijft daarom beschikbaar voor T-cel interactie. 2) CD20 komt in hoge expressie voor op > 90% van de B-cel lymfomen. 3) CD20 komt alleen voor op B-cellen gedurende een aantal stadia van B-cel ontwikkeling, maar niet op stamcellen of volwassen plasmacellen, dus de patiënt blijft op lange termijn het afweersysteem via zijn B-cellen behouden. 4) Binding van BIS20x3 aan CD20 moleculen leidt tot clustering van CD20 moleculen en uiteindelijk tot inductie van apoptose, zelfs zonder T-cel aanwezigheid. 5) In aanwezigheid van T-cellen worden deze zeer efficiënt geactiveerd en geredigeerd naar de B-cel. Deze T-cel activatie is waarschijnlijk zo efficiënt doordat B-cellen met hun natuurlijke arsenaal aan eiwitten in staat zijn T-cellen te costimuleren. 6) Dit costimulatoir arsenaal wordt ook nog eens verder gestimuleerd door specifieke CD20-activatie signalen. 7) Binding van een antilichaam aan CD20 leidt tot translocatie van CD20 naar rafts, een proces dat samen gaat met Fas-clustering op het celoppervlak, vorming van de DISC en de inductie van death-receptor specifieke apoptose. 8) CD20 induceert een toename van Fas expressie op B-cellen en raft afhankelijke Fas-clustering. Hierdoor worden B-cellen gevoeliger voor FasL op T-cellen en gaan makkelijker in apoptose. 9) CD20 kan een rechtstreekse interactie aangaan met HLA-DR moleculen, waarschijnlijk in de context van een groter sigalingsplatform van de MHC klasse II immuun synaps, met meerdere ondersteunende moleculen. Dit creëert een milieu waarin B-cel / T-cel contacten op een natuurlijke manier plaats kunnen vinden. 10) Naast Fas sensitisatie blijkt CD20 ook op andere manieren de gevoeligheid van B-cellen voor apoptose te beïnvloeden: via diverse membraan geassocieerde processen, waaronder calciumgeleiding en de vorming van lipid rafts; via de remming van signalingsroutes als de MAPK-, Akt- ERK- of NF-κB-route en via hun effecten op pro- en anti-apoptose eiwitten zoals die van de Bcl-2 familie. Al deze eigenschappen zorgen ervoor dat T-cellen maximaal geactiveerd kunnen worden en B-cellen optimaal gevoelig zijn voor T-cel cytotoxiciteit.

Wanneer onze kennis over B-cel regulatie en het natuurlijk functioneren van een eiwit als CD20 groeit, groeit hiermee ook onze kans om een beter medicijn te maken tegen B-cel gemedieerde ziekten. De vraag is of een bispecifiek antilichaam als BIS20x3 zich kan meten met de huidige therapieën, zoals rituximab, of andere in ontwikkeling zijnde nieuwe monoklonale antilichamen gericht tegen CD20. In het

verleden werden antilichamen vaak in muizencellen geproduceerd en hebben daardoor muizen-kenmerken. Deze kunnen in de patiënt herkend worden als lichaamsvreemd en een heftige reactie geven (HAMA reactie of human-anti-mouse-antibody reactie). Rituximab is grotendeels humaan en heeft slechts voor een klein deel muizen-kenmerken. Daarom is deze reactie minder vaak aanwezig. De nieuwste trend de afgelopen jaren is de ontwikkeling van volledig humane antilichamen tegen CD20, die optimaal gebruik kunnen maken van het immuunsysteem van de patiënt en niet herkend worden als lichaamsvreemd. De vorming van de zogenaamde HAMA reactie is in het verleden een probleem gebleken voor het gebruik van bispecifieke antilichamen in de kliniek. Hiernaast waren de eerste klinische resultaten met bispecifieke antilichamen helaas matig effectief, met relatief veel bijwerkingen. T-cel activatie lijkt soms erg ongecontroleerd aanwezig en kan leiden tot een extreme productie van ontstekingsfactoren in de patiënt met veel bijwerkingen. Ondanks de veelbelovende resultaten in het laboratorium en in muizen zal BIS20x3 daarom eerst verder ontwikkeld moeten worden voor het in mensen gebruikt kan worden. Bijvoorbeeld door het antilichaam verder te humaniseren of door de controleerbaarheid van T-cel activatie verder te analyseren. Uiteindelijk zou een gehumaniseerd BIS20x3 eventueel toegepast kunnen worden in een pilot-studie met B-cel lymfoom patiënten die uitbehandeld zijn of ongevoelig zijn geworden voor rituximab therapie. Welke plaats BIS20x3 uiteindelijk heeft t.o.v. andere nieuwe, volledig humane CD20 antilichamen zal verder bekeken moeten worden, maar de potentiële effectiviteit van het bispecifieke antilichaam beschreven in dit proefschrift is erg bijzonder en daarom lijkt een vervolg t.a.v. dit werk zeker gerechtvaardigd.

Dankwoord



Dankwoord

Eindelijk is het zover, het laatste hoofdstuk van mijn proefschrift. De eerste stappen voor de totstandkoming van dit werk werden reeds gezet in 1999 toen ik als doctoraalstudent mocht participeren in het onderzoek van de Tumor Immunologie groep op het U-lab van de afdeling Medische Biologie in Groningen. En nu, na zoveel jaren, is het boekje klaar!

Geen enkel proefschrift komt tot stand zonder de hulp, adviezen en stimulatie van anderen. Daarom wil ik in dit hoofdstuk proberen zoveel mogelijk mensen te bedanken die betrokken zijn geweest bij het onderzoek opgeschreven in dit proefschrift (en bij het vele werk dat er niet in staat).

Als eerste wil ik graag mijn copromotor Bart Jan Kroesen bedanken. De begeleiding van een promovendus is essentieel en zonder jou was dit proefschrift er nooit gekomen. We hebben uitgebreid gediscussieerd over experimenten, resultaten (zowel succesvol als mislukt), vervolgexperimenten en nieuwe onderzoeksplannen. Hiernaast liet je me ook de vrijheid om zelf mijn weg te vinden op het onderzoekspad, om met vallen en opstaan uiteindelijk op mijn pootjes terecht te komen. De publicaties werden net zolang bijgeschaafd tot we allebei tevreden waren en ik ben erg blij dat we deze jaren goed hebben kunnen samenwerken. Als bijzonder moment denk ik nog graag terug aan ons gezamenlijk congres in Steamboat Springs, waar we het nuttige met het aangename konden verenigen op de ski's. En waar we met gevaar voor eigen leven in een jeep de berg op zijn gereden naar de Hot Springs, onder een heldere sterrenhemel in de sneeuw, in de bergen van de Rocky Mountains. Heel bijzonder!

Dan vervolgens mijn beide promotores. Hanneke, je hebt me af en toe moeten prikkelen, duwen en sleuren, maar zonder jou had dit proefschrift waarschijnlijk nog steeds op een plank gelegen bij de "nog af te maken" dingen. Ik wil je bedanken voor je blijvende enthousiasme, constructieve begeleiding maar ook voor de af en toe noodzakelijke "draai om de oren".
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Ik wil de leescommissie, bestaande uit Prof. dr. C.G.M. Kallenberg, Prof. dr. J.G.J. van de Winkel en Prof. dr. J.H.F. Falkenburg, bedanken voor het beoordelen en goedkeuren van dit proefschrift.

Professor Falkenburg wil ik hiernaast nog extra bedanken voor de weken die ik in zijn lab door heb mogen brengen in Leiden. Natuurlijk gaat mijn dank hierbij ook uit naar Bart Nijmeijer die mij zijn diermodel "leende" om experimenten met BIS20x3 in te doen en die het proefdierwerk beschreven in hoofdstuk 6 uitgevoerd heeft. Ik vond het heel bijzonder en leerzaam om eens rond te kijken in een ander onderzoekscentrum en wil iedereen op het lab daar bedanken voor de leuke tijd!

Professor Kallenberg, Cees, naast lid van de leescommissie ben je ook het hoofd van de afdeling waar ik nu werk: de afdeling Reumatologie en Klinische Immunologie in het UMCG. Mede onder jouw bezielende leiding mag ik mijn opleiding tot reumatoloog bij jullie doen. Ik hoop dat ik de komende jaren ook op wetenschappelijk gebied mijn steentje kan bijdragen aan de ontwikkeling van deze jonge, bruisende afdeling.

Sebo Withof, ooit kwam ik als student bij je binnen op zoek naar een onderwerp voor mijn doctoraal onderzoek. Later werd ik je eerste AIO. Ik heb veel van je geleerd, op onderzoekstechnisch gebied, bij het uitdenken van experimenten en later ook bij het begeleiden van studenten. Je zit nu alweer een tijdje in de Verenigde Staten. Ik hoop dat het je goed gaat, zowel op wetenschappelijk gebied als in je persoonlijk leven samen met Diana.

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In de loop der jaren heb ik verschillende studenten van verschillende opleidingen (mede) mogen begeleiden. Of jullie nu van de HLO kwamen, via Biologie of via Geneeskunde, iedereen bedankt voor zijn of haar inzet en enthousiasme. En uiteraard bedankt voor alle resultaten die ik heb mogen gebruiken in mijn artikelen Ingrid Drossaart, Vera Kovalchuk, Hisko Oeseburg, Andries van der Meer, Ramune Nedveckyte, Hans van Hateren, Leonie Delahaye, Tineke de Boer, Kor-Johan Koning, Marieke Geel en Wouter Pattje.

De omgeving waarin het wetenschappelijk werk ten uitvoer wordt gebracht is erg belangrijk voor het slagen van experimenten en voor de frisse kijk op nieuwe creatieve ideeën. Een goede sfeer, humor en ontspanning zijn een wezenlijk onderdeel van succes. Dankzij alle etentjes, borrels, labdagen enzovoort was de atmosfeer binnen de Medische Biologie al die jaren uitstekend. Vooral onder de AIO's werd veel gelachen, maar konden de frustraties van het "AIO-zijn" ook gedeeld worden. Ik wil alle (ex)-AIO's van de Medische Biologie en Klinische Immunologie hiervoor bedanken, in het bijzonder mijn kamergenootjes Agnieska, Andre, Edwin, Esther, Hannie, Jan-Stephan, Wayel en Karina.

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Zonder paranimfen is er geen promotie en geen feest. Esther en Leonie dank voor jullie bijstand op deze dag en dank voor jullie hulp bij de voorbereidingen vooraf. Met de andere biologiemeiden werden iedere maand onder genot van lekker eten en drinken alle belangrijke en minder belangrijke zaken doorgenomen en kon er heerlijk gemopperd en gerelativeerd worden. We wonen tegenwoordig door het hele land verspreid, maar ons jaarlijkse weekend houden we in stand!

Altijd in het ziekenhuis zitten, als dokter of als onderzoeker, is ook niet gezond. Gelukkig heb ik daarom nog altijd mijn grote uitlaadklep: volleybal! Door af en toe tegen een bal aan te meppen blijft je hoofd helder en je blijft er fysiek ook nog (een beetje) fit bij! In de kantine en op het veld kan ik mijn teamgenootjes daarom nog

niet missen! Bedankt Miekes voor jullie jarenlange support zowel op het veld als erbuiten! Ook alle andere vrienden in Emmen, Groningen, Klazienaveen, Exloo, Hooghalen en waar ook in Nederland, dank jullie wel voor de nodige ontspanning naast mijn werk.

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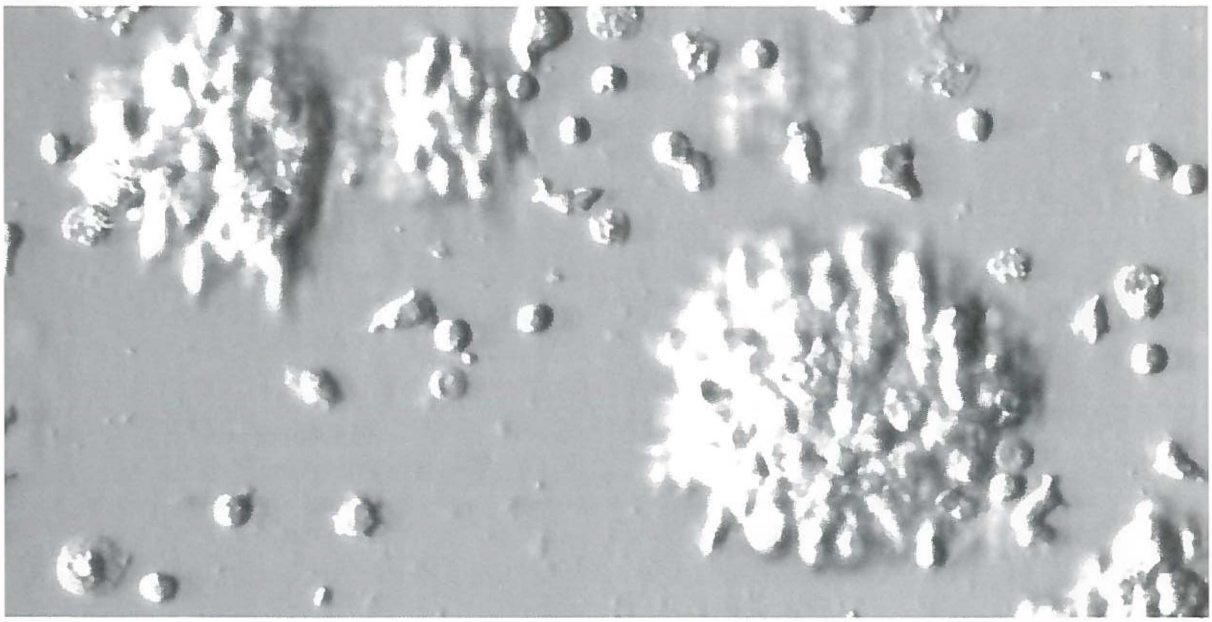
Marco, je bent al jarenlang mijn stille kracht op de achtergrond, dank je wel dat je alles met mij wilt delen.

Abbreviations



Abbreviations used in this thesis

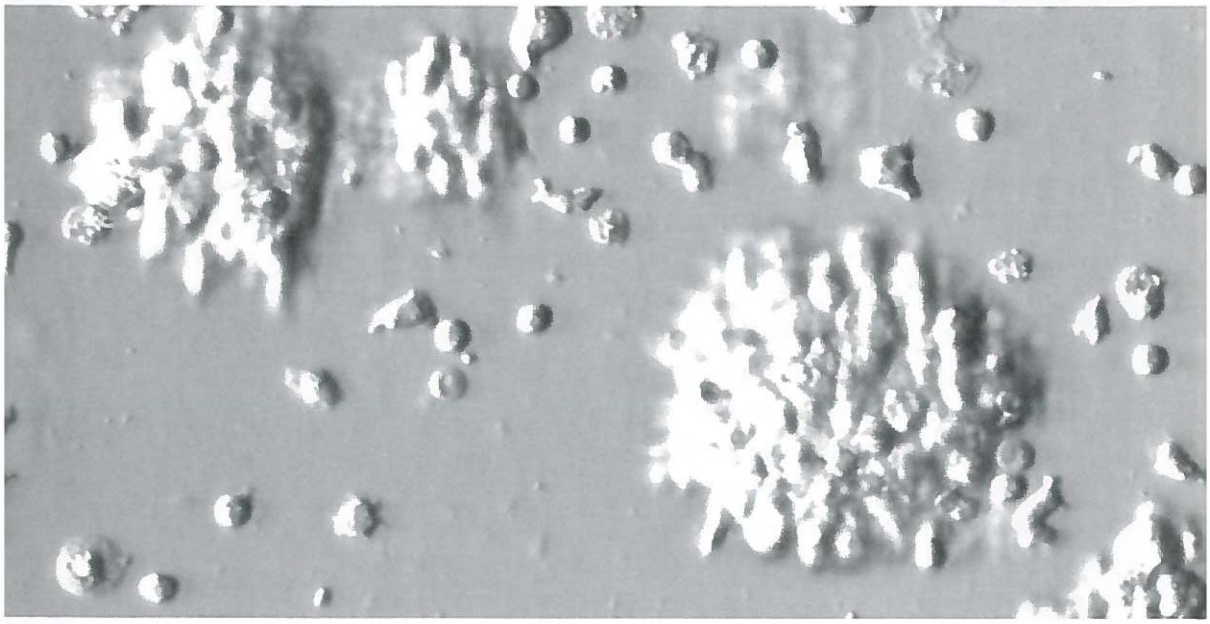
Ab	antibody
AC	absolute (leukemic cell) count
ADCC	antibody dependent cellular cytotoxicity
Ag	antigen
AIDS	acquired immunodeficiency syndrome
ALL	acute lymphocytic leukemia
AP-1	activator protein-1
Apaf-1	apoptosis protease activating factor-1
APC	antigen presenting cell
APC	allophycocyanin
ATCC	American Type Tissue Collection
BcR	B-cell receptor
BLS	bare lymphocyte syndrome
BM	bone marrow
BSA	bovine serum albumine
BsAb	bispecific antibody
cIAP	cellular Inhibitor of Apoptosis Protein
CD	cluster of differentiation
CDC	complement dependent cytotoxicity
CDR	complementarity determining region
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
CIITA	class II transactivator
CL	crosslinker
CLL	chronic lymphocytic leukemia
CML	chronic myeloid leukemia
CMV	cytomegalovirus
CNS	central nervous system
C region	constant region of Ig
CSF	colony stimulating factor
CFSE	carboxyfluorescein diacetatesuccinimidyl ester
CTL	cytotoxic T-lymphocyte
DC	dendritic cell
DFF	DNA fragmentation factor
DISC	Death Inducing Signaling Complex
DKFZ	Deutsches Krebsforschungs-zentrum
DLI	donor lymphocyte infusion
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
EBV	Epstein Barr virus
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol-bis(b-aminoethyl ester)-N,N,N',N'-tetraacetic acid
ER	endoplasmatic reticulum
ERK	Extracellular signal-Regulated Kinase

E/T	effector/target
Fab	antigen-binding fragment
FACS	fluorescence-activated cell sorter
FADD	Fas-Associated protein with Death Domain
FasL	Fas-ligand
Fc	crystallizable fragment
FcR	Fc-receptor
FcεRIβ	IgE Fc-receptor beta-chain
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FLICE	Fas-associated death domain-like IL-1β-converting enzyme
FLIP	FLICE-Inhibitory protein
FPLC	fast protein liquid chromatography
FRET	fluorescence resonance energy transfer
g	relative centrifuge force (gravity)
gr	gram
GaH	goat-anti-human
GaM	goat-anti-mouse
GC	germinal center
G-CSF	granulocyte CSF
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage CSF
GVH	graft versus host
HAT	hypoxantine, aminopterine and thymidine
HEPES	4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid
HGPRT	hypoxanthine-guanine phosphoribosyl-transferase
HIV	human immunodeficiency virus
HLA	human histocompatibility leukocyte antigen
hr	hours
HRP	horseradish peroxidise
HSC	hematopoietic stem cell
HT-supplement	sodium hypoxanthine and thymidine supplement
ICAM	intercellular adhesion molecule
Id	idiotype
Ig	immunoglobulin
INF	interferon
IκB	inhibitory NF-κB
IL	interleukin
i.p.	intraperitoneal(ly)
IP	immunoprecipitation
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
IU	international unit
i.v.	intravenous(ly)
JAK	Janus Kinase
kDa	kilodalton
KO	knockout
L	ligand
LFA	lymphocyte function-associated antigen
LN	lymphnode
LPS	lipopolysaccharide
LUC	luciferase
m	meter

M	molar
mAb	monoclonal antibody
MACS	magnetic-activated cell sorting
MAPK	Mitogen-Activated Protein kinase
µCi	microcurie
MEK	MAPK or ERK kinase
MFI	mean fluorescence intensity
mg	milligram
µg	microgram
MHC	major histocompatibility complex
min	minute
ml	milliliter
µl	microliter
mRNA	messenger RNA
NCC	nucleated cell count
NFAT	Nuclear Factor of Activated T-cells
NF-κB	Nuclear Factor-κB
NHL	non-Hodgkin's lymphoma
NIK	NF-κB-Inducing-Kinase
NK cell	natural killer cell
NOD/scid	nonobese diabetic/severe combined immunodeficiency
NP-40	Nonidet P-40
OD	optical density
OS	overall survival
PARP	poly(ADP-ribose) polymerase
PBL	peripheral blood lymphocyte
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PE	phycoerythrin
PEG	polyethyleneglycol
PFHM	protein-free hybridoma medium
PFS	progression free survival
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
PIPES	piperazine-N,N'-bis(2-ethane sulfonic acid)
PKC	protein kinase C
PLCγ	phospholipase Cγ
PMN	polymorphonuclear leukocyte
PMSF	phenylmethane-sulfonylfluoride
PS	phosphatidyl serine
PTLD	post-transplantation lymphoproliferative disorder
r	recombinant
R	receptor
RaH	rabbit-anti-human
RBC	red blood cell
RIT	radioimmunotherapy
RKIP	Raf-1 kinase inhibitor
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
sec	second
s.c.	subcutaneous(ly)
SCF	stem cell factor

SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SD	standard deviation
SEM	standard error of the mean
siRNA	small interfering RNA
SMAC	supra molecular activation cluster
STAT	Signal Transducer and Activator of Transcription
TAK1	TGF-β Activating Kinase 1
TBS	Tris-buffered saline
Tc	cytotoxic T-cell
TcR	T-cell receptor
Tg	transgenic
TGF	transforming growth factor
Th	T-helper (cell)
TLR	Toll-like receptor
TNF	tumour necrosis factor
TRAIL	TNF-related Apoptosis Inducing Ligand
Treg	regulatory T-cell
Tris	tris(hydroxymethyl) aminomethane
TRITC	tetramethyl-rhodamine isothiocyanate
TUNEL	Tdt-mediated dUTP nick end labelling
U	unit
V region	variable region of Ig
V(D)J	variable (diversity) joining
VCAM	vascular cell adhesion molecule
WT	wildtype
XIAP	X-linked Inhibitor of Apoptosis Protein
YY1	Yin-Yang 1

Curriculum Vitae



Curriculum Vitae

Alja Stel werd geboren op 23 december 1976 te Emmen. Zij volgde het gymnasium op de Christelijke Scholen Gemeenschap te Emmen en behaalde in 1995 haar VWO-diploma. In 1995 startte zij met de studie Medische Biologie aan de Rijksuniversiteit Groningen. In 1997 begon zij hiernaast met de studie Geneeskunde aan dezelfde universiteit. Voor de studie Medische Biologie deed zij twee doctoraal onderwerpen. Als eerste in 1999 "De ontwikkeling van bispecifieke CD20xCD3 antilichamen en single-chains als immuuntherapie voor non-Hodgkin's lymfomen" bij de afdeling Medische Biologie (Tumor Immunologie) van het UMCG te Groningen. En in 2001 "De rol van p21CIP/WAF1 in Cisplatinum gevoeligheid van testis tumoren" bij de afdeling Medische Oncologie van het UMCG in Groningen. In deze periode werd de belangstelling voor het doen van wetenschappelijk onderzoek gewekt en steeds verder aangewakkerd. Uiteindelijk resulteerde dit in september 2001 in een MD/PhD project als vervolg op haar eerste doctoraal onderzoek. Dit project betrof de ontwikkeling van een bispecifiek antilichaam (BIS20x3) en het doen van basaal onderzoek naar het eiwit CD20 op B-cellen. Binnen dit MD/PhD traject werd het doen van wetenschappelijk onderzoek gecombineerd met de laatste twee jaren van haar opleiding tot arts: de co-schappen. Op 26 januari 2006 ontving zij haar artsenbul en in februari 2006 begon zij met de opleiding tot internist in het Scheperziekenhuis in Emmen bij opleider dr. F.G.H. van der Kleij. Vanaf februari 2008 vervolgde zij haar opleiding bij Prof. dr. R.O.B. Gans in het UMCG. Per juni 2009 is zij overgestapt naar de vervolgopleiding tot medisch specialist in de Reumatologie bij opleider dr. H. Bootsma. Deze opleiding vindt plaats binnen de afdeling Reumatologie en Klinische Immunologie van het UMCG, met als afdelingshoofd Prof. dr. C.G.M. Kallenberg.
Alja woont sinds 1996 samen met Marco Meijeringh in Groningen.